Characterization of Flax Germplasm for Resistance to Fusarium Wilt Caused by *Fusarium oxysporum* f. sp. *lini*

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Abstract

Flax wilt is caused by the soil-borne fungal pathogen *Fusarium oxysporum* f. sp. *lini* (*Fol*). The best control method is to develop resistant cultivars to be grown in the field. The objectives of this study were to develop a controlled environment phenotyping method to test flax lines for reaction to flax wilt and screen a recombinant inbred line (RIL) population for reaction to flax wilt under a controlled environment and compare the observations with field evaluations at wilt nurseries. Finally, the inheritance of flax wilt resistance of the RIL population was assessed using phenotypic data from the controlled environment and field test. Four flax cultivars: Bison and Aurore (wilt resistant) and Novelty and Oliver (wilt susceptible), were inoculated with 17 *Fol* isolates under controlled conditions. Plants were assessed on the degree of yellowing, browning and wilting. Disease severity (DS) at 21 and 28 days after inoculation (dai) and the area under disease progress curve (AUDPC) were used to group the isolates. Isolates 65, 81 and 131 were the moderately aggressive and selected to phenotype a RIL population developed by crossing Aurore and Oliver (AO). For all isolates gradual increase in DS and AUDPC among RILs was observed. Similar observations were made in each wilt nursery. Moderate correlations were observed between controlled conditions and wilt field nursery experiments, signifying the importance of controlled condition experimental data as predictors of flax wilt resistance in the field. Inheritance of flax wilt resistance was observed to segregate in a 3:1 ratio, of susceptible to resistant. Thus, this suggested that two independent, recessive genes conferred wilt resistance to the AO RIL population. However, the phenotypic observations indicated the additive effect of minor genes.

This study indicated the significance of identifying the inheritance pattern of wilt resistance in flax populations and highlights the need to locate resistance genes within the genome. This would assist in marker assisted selections (MAS) in flax breeding to reduce

time and labour, and also to incorporate wilt resistant genes to develop flax cultivars resilient to *Fol* pathotypes.

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List of Abbreviations

AAFC	Agriculture and Agri-Food Canada
ALA	α-linolenic acid
ANOVA	Analysis of Variance
AUDPC	Area Under Disease Progress Curve
BC	Backcross
BSA	Bulk Segregant Analysis
CDC	Crop Development Center
CV	Coefficient of Variation
dai	days after inoculation
DH	Double Haploid
DNA	Deoxyribo Nucleic Acid
DS	Disease Severity
Fol	*Fusarium oxysporum* f. sp. *lini* (Bolley) Snyder and Hansen
GM	Genetically Modified
H^2	broad sense heritability
h^2	narrow sense heritability
MAS	Marker Assisted Selection
MB	Manitoba
MS medium	Murashige and Skoog basal medium
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
QTL	Quantitative Trait Analysis
RAPD	Random Amplified Polymorphic DNA
RCBD	Randomized Complete Block Design
RIL	Recombinant Inbred Line
SK	Saskatchewan
SNP	Single Nucleotide Polymorphism
SS	Sum of Squares
SSR	Simple Sequence Repeats

Chapter 1: Introduction

1.1 Background and rationale

Flax (*Linum usitatissimum* L) was domesticated in the Fertile Crescent during the Neolithic revolution, which was the period when the nomadic lifestyle gave way to agriculture. Flax is considered one of the eight "founder crops", which means the first crops to be domesticated in human history (Zohary and Hopf, 2001). For over 10,000 years, flax has been cultivated either to obtain the stem fibre or for the oil in the seed (Kislev *et al*., 2011; Allaby *et al.*, 2005). Fibre is used to make linen and paper, while the oil from the seeds is used in the production of quality linoleum flooring and paints. Though it was not commonly consumed as an edible oil in the past, the importance of flax seed has been recognized in recent years as a functional food promoting health and as an animal feed, because of its high omega – 3 fatty acid content (Kislev *et al.*, 2011; Newkirk, 2008; Growing Flax, 2002).

Canadian farmers have been the leading flax seed producers in the world for over two decades, producing an average of 789,376 tonnes of linseed annually, from 1989 to 2009 (FAOSTAT, 2014). While Canada has ranked first in linseed production in the world since 1993, France has produced the most flax fibre since 2009 at an average of 61,442 tonnes per year (FAOSTAT, 2014). The majority of the flax produced in Canada is grown in Saskatchewan, with production of 492,800 tonnes in 2012/2013 (FlaxStatistics, 2014).

First identified to be caused by a *Fusarium* species [*Fusarium oxysporum* f. sp. *lini* (*Fol*)] in the early 20th Century, flax wilt is one of the most important diseases affecting flax in Canada. The pathogen can infect flax at any growth stage and may result in 100% disease incidence in susceptible cultivars (Kommedahl *et al.*, 1970). The pathogen, which can be either soil or seed-borne, invades through roots and develops in the xylem. Mycelial growth in the xylem interferes with water conduction, thus leading to wilting of the plant (Flax

Council of Canada, 2002; Boyle, 1934). Depending on the plant growth stage, death of seedlings or premature death of plants can occur randomly or in distinct patches throughout the field. Plants grown from cracked or split seeds are identified to be more susceptible to flax wilt than intact seeds (Rashid and Kenaschuk, 1993; Kommedahl *et al.*, 1970). Temperature and moisture level are considered the main factors affecting disease development.

It is difficult to eradicate the pathogen from a field once infested, because the pathogen is able to survive for many years as a saprophyte in plant debris in the soil or by producing dormant, thick walled chlamydospores. The most commonly practiced disease control method is the use of resistant cultivars, together with crop rotation for at least three years which helps lower the inoculum level, thus reducing disease incidence, severity and selection pressure on resistant cultivars (Rashid, 2012; Flax Council of Canada, 2002). Resistance to flax wilt among cultivars differs due to the variability of pathogen pathotypes in different geographical regions together with varying temperatures and environmental conditions (Rashid and Kenaschuk, 1993, Kommedahl 1970).

Flor's study (Flor, 1940) on flax rust was the basis for the gene-for-gene theory based on the observations of flax and *Melampsora lini* interactions. The genetics and resistance mechanisms of flax wilt have never been fully understood, although resistance to the disease was developed by selection and recombination (Kroes, 1997). Diederichsen and Fu (2008), have shown that germplasm from North America and East Asia has above average resistance to flax wilt, compared with accessions from the Indian subcontinent and Europe. None of the existing Canadian flax cultivars show resistance to all isolates of *Fol* collected in western Canada, and resistance has broken down on occasion (Mpofu and Rashid, 2001).

Recombinant inbred lines (RILs) and doubled haploid populations (DH) were developed from crosses between flax wilt resistant and susceptible parents. Early attempts

at identifying major genes conferring resistance using these segregating populations from resistant and susceptible parents were unsuccessful and it was determined that resistance was due to polygenic effects (Kommedahl et al., 1970). However, Spielmeyer *et al.* (1998b) later suggested that major gene effects may be masked by the presence of different pathotypes in the field. Also, segregation of many genes from genetically diverse parents affecting resistance or unreliable infection because of heterogeneity of field conditions can result in quantitative variation. Resistance in a cross between a Linola (linseed variety with high linoleic acid content and low linolenic acid content) line (CRZY8/RA91) and cv. Glenleg was attributed to two major genes, along with minor gene expression, contributing to transgressive segregation of a significant number of resistant doubled haploid (DH) lines (Spielmeyer *et al.* 1998b). However, these contrasting observations might be due to the existence of different races as has been observed with vascular wilt of melon caused by four races of *F. oxysporum* f. sp. *melonis* (Perchepied et al., 2005). Of the four races, two independent genes, *Fom*-1 and *Fom*-2, were observed to provide resistance against Races 1 and 2, while both genes were resistant to Race 0. However resistance towards Race 1.2 was observed to be polygenic.

Screening for *Fol* resistance is based on visual assessment. In the field this can generate variable results due to environmental effects and interactions with soil microflora, and would require many replications in time and space to draw a rational conclusion on the inheritance of resistance (Kroes et al., 1998). Experiments conducted under controlled conditions in growth chambers or glass houses, or *in vitro* studies, reduce most of these unpredictable and complicating factors. This generates more stable conditions to study the inheritance of resistance of complex plant – pathogen interactions between flax and *Fol* (Spielmeyer et al., 1998b). They observed significant correlation between wilt nursery and

glasshouse experiments in their study, and concluded that controlled environment screening can be used as a reliable predictor of field performance.

Many experiments conducted to identify the race structure of *Fol* concluded the existence of an indefinite number of races or pathotypes (Kroes, 1997; Kommedahl et al, 1970), making it difficult to develop a uniformly resistant flax cultivar. Identification of flax genes or quantitative trait loci, conveying resistance to a wider range of *Fol* pathotypes is essential, since it would make it possible to develop a flax cultivar adapted for resistance against different pathotypes of *Fol* existing at different locations.

In this study, a controlled environment screening method was optimized and used to determine the interaction between 17 *Fol* isolates and four flax cultivars. Three of the isolates were used to screen a RIL population in the growth chamber to reduce the environment effects as well as to minimize interactions among strains of the pathogen, as might occur in the field. Field experiments and growth chamber studies were compared to determine the consistency and reliability of the two environments. Finally, observations from both studies were used to determine the inheritance of resistance to *Fol* using a recombinant inbred line population.

1.2 Hypothesis

a) Resistance to flax wilt is conferred by a dominant gene consistent with the gene-for-gene theory

1.3 Objectives

1) Develop a phenotyping method to assess flax cultivars for flax wilt severity under controlled environment,

2) screen a RIL population for flax wilt under controlled environment and compare the observations with field test, and

3) determine the inheritance of flax wilt resistance in the RIL population using the phenotypic data

Chapter 2: Literature Review

2.1 Flax – Classification and History

Flax is an annual plant that belongs in the genus *Linum* and cultivated flax is known as *L. usitatissimum* subsp. *usitatissimum* L. Although approximately 300 species belong in the family Linaceae with nearly 180 annual and perennial species belonging to the genus *Linum*, only cultivated flax is economically important (Wang et al., 2012). Cultivated flax is believed to be closely related to the wild flax species *Linum bienne* found in the Mediterranean, Near East and Western European countries. Cultivated flax is of two types: one grown for seed and the other for fibre. Cultivars grown for both oil and fibre are termed dual purpose. Initially, the same genotype was used for both oil and fibre production, but currently there are specific cultivars for each purpose. In North America only the linseed cultivars are grown for commercial purposes (Pengilly, 2005; Przybylski, 2005).

Flax is one of the first domesticated plants and is considered a foundation crop. The centre of origin of cultivated flax is still debated with speculation that it might be the Mediterranean or Southeast Asia (Jhala and Hall, 2010). The highest species diversity within the genus *Linum* is found in the Indian subcontinent. However, there is speculation that it originated from western Persia and then spread to other countries where flax was first cultivated. It was first introduced into North America by Lois Hébert in 1617 (Cullis, 2007). Three geographical regions of diversification for *Linum* have been suggested: the Mediterranean, southern North America and Mexico, and South America, with most species reported from North America and Mexico (Duguid, 2009). This is considered an indication of separate diversification processes that occured at each geographical location since their distribution from the Near East (Harlan, 1971).

Evidence suggests that flax was cultivated in the Fertile Crescent around 7,000 B.C. E. and linen clothes made of flax fibre were worn in Egypt as early as 5,000 B.C. E. and in

Babylonia in 2,000 B.C. E. (Vaisey-Genser and Morris, 2003). The first use of linseed oil was for embalming in Egypt in 1400 B.C. and flaxseed was used in bread in Jordan and Greece in 1000 B. C. E. However, in a recent excavation conducted in Georgia, wild flax fibres estimated to be over 30,000 years old, were discovered in a prehistoric cave, indicating the early use of flax (Kvavadze et al., 2009). Flax was introduced to North America almost 400 years ago and commercial production increased considerably after the Second World War. Eventually Canada became a leader in flax production (Pengilly, 2005).

2.2 Flax Production

According to Food and Agriculture Organization (FAO) statistics, average linseed production in Canada in 2013 was 712,000 t, which accounted for nearly 21% of world linseed production. China was second at 369,000 t, less than half the Canadian production. The Russian federation and Kazakhstan were the other leading linseed producers (FAOSTAT, 2014). Seventy to eighty percent of Canadian linseed is produced in Saskatchewan, with the remainder from Manitoba and Alberta (FlaxStatistics, 2014). France was the leading fibre flax producer in the world with production of approximately 83,100 t in 2013, followed by Belgium, Belarus, the Russian Federation and China as the top fibre flax producers (FAOSTAT, 2014).

Nearly 80% of Canadian linseed is exported, with about 70% shipped to the European Union (EU), and the rest exported to the United States of America (US), China and Japan (Viju et al., 2011). Canadian flax production averaged approximately 789,000 t over the two decades from 1989 to 2009 and has increased from ~500,000 t to ~930,000 t during this period (FAOSTAT, 2014).

The presence of a low level of genetically modified (GM) flax cultivar, CDC Triffid (a sulfonylurea herbicide-resistant cultivar) (McHughen et al., 1997), was detected in shipments to Europe+ in 2009. The Europeans maintain a zero tolerance policy on GM flax, effectively ending Canadian linseed shipments to Europe, which resulted in a reduction of linseed production in 2010 and 2011 (Viju et al., 2011). To demonstrate compliance, the Canadian industry adopted a protocol involving testing grain samples (post-harvest) using an RT-PCR test for the construct found in CDC Triffid. Samples of flax are collected based on the weight of flax grain lots entering the system (Canadian Grain Commission, 2015). Two kg composite samples are collected and four 60 g subsamples are subjected to a real-time polymerase chain reaction (PCR) assay. This provides 0.01% level of detection (1 in 9999 seeds), although clean seed lots can result in a positive test due to the PCR assay error rate and repeated tests (Lamb and Booker, 2011; Booker and Lamb, 2012, Booker et al., 2014). An event-specific quantitative PCR (qPRC) protocol was developed by Young et al. (2015) to determine the genetics and occurrence of the transfer DNA (T-DNA) in CDC Triffid at levels between 0.01 and 0.1%. Continued testing, production and seeding of commercial cultivars free of Triffid, has restored the market for Canadian flax (Booker et al., 2014). Together these efforts resulted in the establishment of a more balanced market among EU, USA and China, and linseed production increased almost to the pre-Triffid incident level in 2013 (FAOSTAT, 2014).

2.3 Morphology of Flax

Flax is a diploid (2n=30), annual, self–pollinating plant with a genome size of approximately 370 Mb (Cloutier et al., 2012) and an outcrossing rate of 0.6 – 2% due to insects (Spielmeyer et al., 1998b).

Fibre and linseed flax differ in several aspects, including morphology, method and time of harvest and industrial uses. Linseed cultivars are shorter (60 - 80 cm) with more branches bearing more seed capsules with larger seeds (thousand seed weight [TWS] of 5.4 - 14 g), whereas cultivars grown for fibre can be 80 - 120 cm tall, with less branching, fewer seed capsules and smaller seeds (TSW < 5.4 g), and a more developed root system (Heller et al., 2014; Przybylski, 2005). Plant height varies with soil fertility, plant density and soil moisture, in addition to the type and cultivar.

The life span of the flax plant varies from 90 to 125 days, which can be divided into three main stages: vegetative, flowering and maturation. The flax plant has one main stem, from which branches can develop under low plant density and high nitrogen fertility.

Flax has a shallow, branched tap root system. Flowers can be blue, white or pale pink, with blue or yellow coloured anthers and last less than 24 hours. The fruit of flax is a capsule and the seeds can vary in colour from yellow to brown or nearly black. While yellow seed is in greater consumer demand, the seed coat of most registered cultivars in Canada is dark brown (Oplinger et al., 1997; Growing Flax, 2002; Przybylski, 2005). Yellow seed coat was required for low (<5 %) linolenic acid flax in Canada, known as solin (trade name LinolaT^{M}) cultivars (Mittapalli and Rowland, 2003) but these were deregistered in 2013 (Annonymous, 2012). Recently, yellow seed coat cultivars with the traditional high linolenic (>50%) acid profile have been developed for production in Canada (Flax Council of Canada, 2015).

2.4 Properties of Flax

Flax seeds contain about 41% fat, 28% dietary fibre, 20% protein and 7% carbohydrates by weight (Oplinger et al., 1997; Przybylski, 2005). Most of the fatty acids in flax are polyunsaturated (nearly 73%), while 18% are present as monounsaturated and only 9% are

saturated. Flax oil is considered healthy because of its high α-linolenic acid (ALA) content (about 50% of the total fat), with less linoleic acid (18% of the total fat). ALA forms omega-3 fatty acid and linoleic acid is the precursor of omega-6 fatty acid, both of which are considered essential fatty acids. Flax seed also contains minerals, such as potassium, phosphorous, magnesium and calcium, and several vitamins, such as niacin and small amounts of Vitamin B6 (Pengilly, 2005).

2.5 Flax Cultivation

Flax is widely grown around the world as a minor crop, possibly due to its high adaptability and diverse uses. In Canada, flax is planted in May or early June and harvested in September. Relatively low temperatures in Canada combined with a long photoperiod during the summer, promotes high oil content in flax seed. However, flax is observed to flourish at higher temperatures of approximately 24˚C in Argentina (Sosulski and Gore, 1964). Flax grown under long photoperiod and cool temperatures has high oil and linolenic acid content and iodine number. The amount of precipitation can also influence flax yield or oil content, especially the amount of moisture that plants receive during the period from flowering to maturity (Oplinger et al., 1997).

Length of photoperiod is vital for flax growth, flowering and maturation, with long days promoting plant growth and reducing the flowering time. This is of special importance in the northern prairies of Canada, where time to maturity is a limiting factor, since late frost in the spring or early frost in the fall can damage the plants, significantly reducing the yield (Sun, 2015).

Flax can be grown in a range of soils although fertile, fine-textured, clay soils with proper drainage are preferred, rather than coarse textured, sandy soils. Flax can be used as

a companion crop because it has limited leaf area and short stature, a shallow root system and early maturity (Heller et al., 2014; Oplinger et al., 1997).

Harvesting of flax differs between oil and fibre types in terms of time and method. The seed ripe stage (Agriculture and Agri-Food Canada, 2011), where seeds are brown and stem is green/ yellow with upper branches senescing, is the ideal time to harvest fibre flax, while full maturity of the seeds is preferred for oilseed flax (Heller et al., 2014).

2.5.1 Flax Breeding

In Canada, the Crop Development Center (CDC) at University of Saskatchewan, Saskatoon, SK is currently the only institution involved in flax varietal development (Flax Council of Canada, 2015). The major focus of early flax breeding programs was on yield increase and disease resistance, specifically to rust and wilt (Cullis, 2007). The objectives of the current program are to reduce maturation time and enhance quality and yield, while incorporating disease and lodging resistance. This has been challenging, since most of these desired characteristics in flax have been optimized (Cullis, 2007). Many of these characteristics are polygenically inherited or have low heritability (Marshall, 1992). Since it is an inbred plant, hybridisation can be used to induce genetic variation followed by selection methods (single seed descent, pedigree, mass and marker assisted) to select desirable recombinants.

Breeding a dual purpose flax cultivar with the ability to produce high seed quality and high fibre yield, is the next step in flax breeding (Soto-Cerda et al., 2013). However, this is difficult, since the requirements for oil and fibre producing cultivars are different. Also, more variation is observed among linseed cultivars (oil producing) compared to flax cultivars (fibre producing) and higher genetic variation is achieved by linseed x linseed and

linseed x fibre flax crosses, than fibre x fibre crosses (Foster et al., 1997). Flax line FP944 (Klass) is a cultivar bred for both fibre and seed (Cullis, 2007).

Development of doubled haploid (DH) and recombinant inbred lines (RIL), are used to ensure homozygous populations from a cross between two parents carrying desirable characteristics. In the doubled haploid method, chromosome number is doubled, producing a homozygous population in one generation. To develop a RIL population, a single seed is selected from each plant of F_2 or F_3 generation onwards, so that by the F_8 generation the population is comprised mostly of homozygous individuals. A segregating homozygous population is beneficial in genetic mapping, and a RIL population is more advantageous in comparison to DH, since selection occurs through more generations which enables more recombination events (Broman, 2005).

Mutation breeding and genetic modification has been attempted to develop flax lines with novel traits. Green and Marshall (1984) subjected the seeds of Australian flax cultivar Glenleg, to different levels of gamma radiation or ethyl-methane sulphonate (EMS) to induce mutation. The mutant lines had low ALA (29%) and high linoleic acid (30%), increasing the shelf life of flax-seed oil, since high ALA is susceptible to oxidation causing rancidity. The first registered solin cultivar was Linola ^{TM} 947; and it had a very low level of ALA (2%) with yellow seed coat for identification (Dribnenkil & Green, 1995). Low ALA was attributed to two recessive alleles resulting from a double mutation (Rowland, 1991). The first genetically modified flax cultivar, CDC Triffid was developed using *Agrobacterium* mediated transformation (McHughen et al., 1997). To develop CDC Triffid, NorLin was inoculated with *A. tumefaciens* carrying the acetolactase synthase gene from *Arabidopsis*, which conferred resistance against sulfonylurea herbicides. However, solin cultivars and CDC Triffid were both deregistered, the former because of limited shelf

life (Annonymous, 2012), the latter due to concerns about production of genetically modified flax affecting the export market (Booker et al., 2014).

2.6 Uses of Flax

Flax was an important oil and fibre crop in ancient civilizations (Zohary and Hopf, 2001) and was used mainly to make linen, a comfortable and long lasting fabric (Muir and Westcott, 2003). It was the main fibre used in textile production until the mass production of cotton and synthetic fibre, in the twentieth century, when the demand for linen decreased (Zohary and Hopf, 2001).

At present, flax fibre is a major component of linen, and is used in the production of recycled paper, to strengthen the paper. The possibility of using whole flax straw as a biofuel is under study (Duguid, 2010). In addition, flax fibre can be used to insulate walls (Jhala and Hall, 2010) and ceilings of houses and buildings in the same way as fibreglass (Flax, 2012).

Flax seed oil was used in Egyptian times for embalming and there is evidence that it was used as a medicine. Linseed oil was used in paint and used extensively for flooring material, which was known as linoleum. Residue left after oil extraction has high levels of protein (30 - 40%) and 3 - 4% fat, similar to whole seeds, making it a high value animal feed. Adding flax to animal feed is recognized to increase the healthy fat content in meat and dairy products (Jhala and Hall, 2010; Muir and Westcott, 2003).

Since ancient times, whole or milled flax seed has been consumed. Sometimes, it is added to baked food or used as an edible oil source due to its high nutrient content. In the recent years, the importance of flax seed and oil as a functional food has been studied, for its high omega 3 fatty acid, fibre and lignin content and antioxidant properties. The potential to use flax seed or oil to reduce the risk of cancerous tumors and to lower blood

pressure are important (Jhala and Hall, 2010; Oomah and Mazza, 1999; Growing Flax, 2002). Recently, Health Canada approved the claim that linseed consumption lowers blood cholesterol (Health Canada, 2014).

2.7 Diseases of Flax

The major diseases of flax that result in significant yield loss are flax rust, caused by *Melampsora lini* (Ehrenb.) Lev., flax wilt, caused by *Fusarium oxysporum* f. sp. *lini* (Bolley) Snyder and Hansen and pasmo, caused by *Septoria linicola* (Speg.) Garass. (Ehrensing, 2008). Control of flax diseases has been achieved, mainly by conventional control methods such as crop rotation, cultivation of resistant cultivars, certified seed use and fungicide application (Rashid, 2003). Resistance to flax rust is maintained to date by cultivation of resistant cultivars carrying one or more combinations out of 34 locus/allele combinations at 7 different loci (K, L, M, N, P, D and Q), each combination segregated as a closely linked group of alleles/genes (Dodds et al., 2006; Rashid and Kenaschuk, 1999; Islam and Mayo, 1990; Flor, 1965). Rust resistance has been a requirement for Canadian flax cultivars for decades, with seven alleles of the K, L, M groups that confer immunity to flax rust (race 371) present in all flax cultivars registered for production in Canada.

The most prevalent disease of flax in Canada in 2013 was pasmo, reported in 86% of the 92 of the fields surveyed (Rashid, et al., 2014). Pasmo severity in 2013 was low, although it increased towards the end of the season. Flax wilt was observed at trace levels to 5% in 56% of the fields. While flax wilt severity and incidence on the Canadian Prairies in the past decade is low, 80 - 100% yield loss can result if susceptible cultivars are grown repeatedly in the same field (Rashid, 2003).

2.8 Flax Wilt (Fusarium Wilt of Flax)

2.8.1 Causal Agent

Wilt has been associated with flax since early cultivation. It was first noted by Pliny in the first century, as scorching of the ground where flax was grown for successive seasons, leading to deterioration of soil (Boyle, 1934). Early work on fusarium wilt of flax was conducted by Otto Lugger in 1889, but Hiratsuka (1893) and Bolley (1901) were the first to identify the causal organism to be a *Fusarium* species. The causal agent was first named *F. lini* by Bolley, but was changed to *Fusarium oxysporum* f. sp. *lini* (Bolley) Snyder and Hansen (*Fol*) in 1940 (Kommedahl et al., 1970).

Fusarium oxysporum is one of the most ubiquitous fungal pathogens in the world affecting many important crops. While another wilt caused by the pathogen *Verticillium dahlia has* a wide host range similar to *F. oxysporum,* it does not show the limited host specificity of *F. oxysporum* (Puhalla, 1985). Therefore, the host range of *Fol* is limited to linseed and fibre flax.

Fol is a facultative parasite which can be soil or seed-borne in susceptible cultivars, and can survive indefinitely in crop residues in the absence of the host (Wilson, 1946). Due to the saprophytic nature of the pathogen, it can survive in soil for prolonged periods in the absence of the host and was identified as a strong colonizer of soil organic matter (Kommedahl et al., 1970).

As a soil fungus, it has the ability to survive through various means, one of which is its high capacity for change, morphologically as well as physiologically, to adapt to new environments. The pathogen is believed to be spread with wind-blown soil, irrigation water and contaminated seed (Muskett and Colhoun, 1947).

Fusarium oxysporum f. sp. *lini* produces three types of conidia: small micro conidia; longer, sickle shaped macro conidia; and chlamydospores, a type of resting spore. Soil

inoculated with both micro conidia and chlamydospores did not show change in conidial densities, though 39% of the micro conidia were converted into resistant chlamydospores (Couteaudier and Alabouvette, 1990). More importantly, chlamydospores have a high germination rate and cause severe disease even at low spore concentrations compared to micro conidia. This observation is very important since *Fol* chlamydospores were reported to survive for 50 years in a field without a host (Houston and Knowles, 1949).

2.8.2 Pathogen Infection Process and Host Defense Mechanisms

The *Fol* fungus can attack the plant at any growth stage, with the seedling stage the most susceptible (Kommedahl et al., 1970). Infection occurs through the roots and the pathogen colonizes the xylem, ultimately obstructing water movement, resulting in wilting. Emergence of susceptible cultivars in infected soil is reduced and the majority of the emerged plants will wilt and die before maturity (Kommedahl et al., 1970; Rashid and Kenaschuk, 1993).

The *formae speciales* of *Fusarium oxysporum* are normally hemibiotrophs, initially surviving on live hosts and eventually killing the infected cells (Ma et al., 2013). The mycelia of *Fol*, are observed to grow within and between undifferentiated cells (intercellular and intracellular) in the roots such as the apical cells in root tips. However, differentiation of some host cells caused destruction of the mycelia (e.g. in cork cells), while the others became infected cells. This led to two proposed theories of pathogen infection: first, growing intercellularly through the cortex, then colonizing cortical cells and developing radially through the endodermis to reach the xylem; or entering through the root apex into undifferentiated xylem precursor cells and then into the xylem (Kroes et al., 1997; Turlier and Alabouvette, 1994).

Infection has been observed to take place at three sites: 1) near the root apex, at the site of cell differentiation, 2) at the cell elongation zone, and 3) through root hairs and the epidermis in the older part of the root (Turlier and Alabouvette, 1994).

Apposition build-up next to penetration hyphae is reported to be triggered by the infection process in an attempt to restrict the pathogen. This is followed by cell death or production of multiple cell layers (Kroes et al., 1997). The root tip was colonized and the cortical region was degraded 8 - 16 days after inoculation. Roots were hollowed out after severe colonization and the *Fol* pathogen could be observed approximately 10 mm from the root tip during the 16-day observation period. Differences in resistant and susceptible cultivars were demonstrated in terms of the amount of nonhydrolyzable material in cortical cells and the ability of cortical cell walls to resist hydrolysis by sulfuric acid (Boyle, 1934).

In a similar study to understand the physiological responses related to infection with pathogenic and non-pathogenic *Fol* strains, H_2O_2 production and Ca^+ flux was documented to be closely related to *Fol* infection (Olivain et al., 2003). Death of infected cells was reported, though the longevity of infected cells was higher with aggressive (pathogenic) strains.

2.8.3 Symptoms of Flax Wilt

Disease symptoms of flax wilt vary depending on the growth stage of the plant, cultivar, environmental conditions and pathotype of the pathogen. If infection occurs early in the plant life cycle, disease can be severe and may result in 100% plant mortality. Seedlings may die even before emergence, or the plants may collapse after emergence. Later, wilt can result in yellowing of leaves, premature ripening and reduced number of seeds per boll or plant, leading to yield loss and premature plant death. At the base of the stem of infected plants, white mycelial growth can be observed, especially under humid conditions. Roots

of the dead plants appear ash-grey (Rashid and Kenaschuk, 1993; Kommedahl et al., 1970; Muskett and Colhoun, 1947).

In seedlings, growth ceases when the leaves and the stem die in response to infection. In older seedlings or young plants, the leaves may turn brown and the top of the plant may turn down to form a “shepherd’s crook”, although the plant remains erect. Plants may wilt and die from the top down, regardless of time of infection during the flax life cycle (Muskett and Colhoun, 1947).

In some cases, partial wilt is observed, where a seedling dies except for the roots and buds. If weather becomes more suitable for the plant than the pathogen, then the buds may form lateral branches and the plant may continue to thrive. A similar exception is unilateral wilt, where only one side of the stem, and the branches on that side, exhibit wilt symptoms due to pathogen colonization of the xylem elements (Rashid, 2003; Kommedahl et al., 1970).

(a) (b)

Figure 2.1 a) Two flax seedlings showing partial wilt, with infected main stem and healthy lateral stem, b) unilateral wilt of a flax plant where leaves on one side are wilted, while the other side is disease free.

2.8.4 Environmental Effects on Flax Wilt

Flax wilt severity is correlated with temperature (Wilson, 1946). Tisdale (1916) observed that environment, particularly temperature, was the most important factor in flax

wilt disease development. Even the most resistant cultivars wilted at high temperature, while even the most susceptible did not wilt at low temperature (Tisdale cited in Sherbakoff, 1949). The optimum temperature for the pathogen to grow in the soil is considered to be 24 - 28˚C, although it can survive over a wide range, 14 - 38 ˚C (Saharan and Mehta, 2005; Muskett and Colhoun, 1947).

According to Kommedahl et al. (1970), the incidence of wilt increased at low soil moisture (dry soil), which enhanced the effect of the pathogen and was assumed to be due to favorable conditions for antagonistic organisms in the rhizosphere. A higher percentage of bacteria with the ability to synthesize growth-promoting substances was found from the root surface of the wilt susceptible flax cultivar 'Novelty', while bacteria requiring growth promoting factors was observed from the rhizosphere of the wilt resistant cultivar 'Bison', indicating differences in soil microflora with respect to wilt resistance (Lochhead and Cook, 1961).

In studies in Minnesota, it was found that disease incidence in peat soil was low and the addition of nitrogen, potash or phosphate reduced disease incidence in susceptible plants (Kommedahl et al., 1970). Sandy soils promote flax wilt, while acidic pH and availability of Ca, Mg and Fe iron to the pathogen all provide a conducive environment (Hoper et al., 1995). Crop loss due to wilt disease can vary from negligible to 100%, depending on these conditions.

2.8.5 Pathogen Pathotypes

Isolates of *Fol* have been identified to vary in: 1) morphology, with respect to the amount and type of sporulation, production of different types of conidia, size and number of septa and pigment production on growth media; 2) physiology, rate and type of growth on substrates and in host; 3) environmental preferences, antibiotic capabilities; and 4)

pathogenicity. Therefore, it is considered an ideal pathogen to demonstrate diversity within a species with numerous biotypes and pathotypes (Kommedahl et al., 1970; Saharan, *et a*l., 2005).

In a study by Broadfoot and Stakman (as cited in Kommedahl et al., 1970) the occurrence of distinct parasitic races was observed on four flax cultivars, based on different characteristics in culture and in morphology of conidia. Also, when a specific cultivar was inoculated with numerous pathotypes of the pathogen, a wide range of resistance or susceptibility was reported indicating the occurrence of races and their antagonism among one another. Ten races of *Fol* were identified in India, based on pathogenicity on eight differential flax cultivars (Saharan et al., 2005).

Vegetative compatibility determines the ability of different strains of a fungus to form heterokaryons, from which the resulting strain might differ in pathogenicity and host range compared with the original individuals (Leslie, 1993). Two vegetatively compatible strains belong in the same vegetative compatibility group (VCG); Elias and Schneider (1991) found 4 VCGs in *Fusarium oxysporum* f. sp. *lycopersici* cause of wilt in tomato (which has 3 races).

Mpofu and Rashid (2001) examined genetic variation of *Fol* using nitrate non-utilizing mutants. They reported 12 vegetative compatibility groups among 74 isolates, while 22 were not assigned to any VCG, which suggested that there is a considerable amount of genetic diversity within *Fol* giving rise to minor differences in pathogenicity. They also noted that each of the six wilt nurseries used in the study, had a different major VCG, which were less frequent or absent in other wilt nurseries indicating either adaptive variation or genealogical differences (Mpofu and Rashid, 2001). It has been reported that *Fol* has multiple independent origins, suggesting the existence of multiple races and VCGs supporting this theory (Baayen et al., 2000).

A limited number of races were reported in other *formae speciales* of *F. oxysporum* causing wilt in different crops. Eight races have been reported to cause fusarium wilt in chickpea (*Cicer arietinum*) and cotton (*Gossypium* spp.), with two distinct pathotypes identified for chickpea resulting in yellowing and wilting (Arvayo-Ortiz et al., 2011; Ulloa et al., 2013). Arvayo-Ortiz et al. (2011) and Assigbetse et al. (1994) noted the restricted geographical distribution of *F. oxysporum* f. sp. *ciceris* (chickpea) and *F. oxysporum* f. sp. *vasinfectum* (cotton) races, which is similar to *Fol*. Three races each have been associated with fusairum wilt of banana and tomato, economically important diseases in both crops (Simons et al., 1998; Li et al., 2013). Race-specific resistance was observed for Race 4 of *F. oxysporum* f. sp. *vasinfectum*, cause of cotton wilt and Race 2 of *F. oxysporum* f. sp. *lycopersici* cause of tomato wilt (Ulloa et al., 2013; Simons et al., 1998). Use of Random Amplified Polymorphic DNA analysis was suggested to distinguish races among *F. oxysporum* f. sp. *vasinfectum*, which could be applicable in *Fol* as well (Assigbetse et al., 1994).

2.8.6 Control of Flax Wilt

The best control method for flax wilt is the cultivation of resistant cultivars in rotation with other crops, with at least three years between flax crops. According to Rashid and Kenaschuk (1993) the commercial cultivars grown in the United States and Canada show moderate resistance towards flax wilt, while the same was observed in Europe (Marshall, 1992). Kommedahl et al. (1970) reported that no flax cultivar had complete resistance to all *Fol* races. Flax lines at the F_8 generation in breeding programs at Agriculture and Agri-Food Canada, with a wilt score of four or less are considered resistant in wilt nurseries at Morden and Indian Head (Mpofu and Rashid, 2001).

North American flax cultivars Bison, AC Linora, AC Emerson, Hanley, Lightning, Macbeth, Prairie Thunder and Shape were reported to exhibit a high level of resistance to flax wilt (Kommedahl et al., 1970; The Western Committee on Plant Diseases, 2012). Resistance to flax wilt is believed to be polygenically inherited and it is considered possible for resistance to breakdown due to the presence of different races/ pathotypes and variation for virulence among regions (Marshall, 1992).

Seed treatment using fungicides such as chloroalkythios, dithiocarbamate and carbendazim is recommended to reduce disease development from infected seed (Saharan et al., 2005). The fungicides dithiocarbamate and tolylmercury acetate were used as seed treatments on wilt resistant and susceptible flax cultivars and it was found that even resistant cultivars could benefit from seed treatment (Wilson, 1946).

Avoiding early seeding in acidic soils is suggested to reduce disease development (Saharan et al., 2005). Acidic, sandy soils provide a conducive environment for flax wilt, resulting in high disease severity. Increasing soil pH by liming (addition of $CaCO^3$) was observed to reduce disease severity along with the addition of clay minerals montmorillonite and illite, which changes soil texture. Iron availability and sand content were negatively correlated with soil suppressiveness of *Fol* (Hoper et al., 1995). Flooding of infected fields may also reduce pathogen inoculum and may reduce disease severity (Saharan et al., 2005).

Trifluralin, a soil incorporated herbicide, was identified to lower post-emergence disease incidence and late wilt (Rashid and Kenaschuk, 1993). However, trifluralin affected the emergence of flax seedlings, especially in fields with high disease incidence. Thus, if the field is known to have severe flax wilt, it is not recommended to use trifluralin, as yield could be greatly affected due to reduced seedling emergence (The Western Committee on Plant Diseases, 2012).

High flax wilt severity was observed in conducive soils with low inoculum, while less disease developed in suppressive soils with high inoculum levels (Scher and Baker, 1980). Therefore, soil amendment is another avenue for the control of flax wilt. In one study, loamy soil amended with compost suppressed flax wilt and the effect was observed to be proportional to the compost application rate. The combination of heat-treated compost and soil increased the half-life of the flax population grown, in comparison to untreated or heat-treated soil, indicating the effect of soil microflora in suppressing *Fol* (Serra-Wittling et al., 1996).

The antagonistic effect of non-pathogenic strains of *F. oxysporum* f. sp. *lycopersici* on pathogenic strains due to competition for nutrients and infection sites on roots and induced resistance in tomato plants infected with non-pathogenic strain Fo47 have been reported to contribute to the control of wilt disease in tomato (Fuchs et al., 1997; Alabouvette and Couteaudier, 1992). Further studies on utilization of non-pathogenic strains of *Fol* in biological control of wilt in flax could be useful.

A mixture of agricultural and aquacultural wastes, such as rice husks, bagasse (residue left after juice extraction from sugarcane) and oyster shell powder, was capable of flax wilt control in a number of crops, when combined with fertilizers to enhance plant growth (Sun and Huang, 1985). In that study, incidence of watermelon wilt, caused by *F. oxysporum* f. sp. *neveum*, was observed to be reduced between 57% - 84% in the field by this mixture, depending on the pathogen propagules present in the soil. Disease control was attributed to inhibition of spore germination and promotion of germ tube lysis of the pathogen, suppression of pathogen growth due to increased pH, and the increase in other soil fungal populations (Sun and Huang, 1985).

The use of anther culture to produce disease-free flax plantlets with increased resistance to wilt was studied by Rutkowska-Krause et al. (2003). They observed that the second

generation of regenerants of anther culture from the Polish flax cultivar Alba, had a significantly higher level of resistance compared to the controls.

2.8.6.1 Host Resistance and Genetic Diversity

Effective control of flax wilt has been attributed to selection of resistant cultivars. Bison, released in 1925 (Thompson and Zimmer, 1943), is a cultivar that has maintained resistance since release and has been a progenitor of many wilt resistant cultivars (Ausemus, 1943). The effect of flax wilt on cultivars was determined by combining seeds from 10 cultivars and cultivating these in different soil types (Kommedahl et al., 1970). These were composed of 40% yellow seeded and 60% brown seeded plants. After one season under high disease severity, yellow seeded plant content dropped from 40% to 5%, and after 4 years, only 4 of the initial 10 cultivars survived, with Bison and CI 671 making up 89% of the surviving plants, indicating natural selection for resistant cultivars in the field.

Although flax is self-pollinated, the cultivars are not genetically uniform. When flax was grown in *Fol* contaminated soil, disease severity of the cultivar Argentine selection (GI 712) differed from 3% to 50% (Kommedahl et al. 1970). Early selection can help to identify resistant lines in a breeding program. The possibility of using *in vitro* assays with single isolates, to screen for wilt resistance in early breeding lines of flax was suggested by Kroes et al. (1998). These *in vitro* results, which included two tests with vermiculite supplemented with 10% Murashige - Skoog medium (MS medium) and liquid medium of only 10% MS medium in test tubes, were significantly correlated with observations from field studies, thus supporting the hypothesis.

Plant Gene Resources of Canada (PGRC) preserves approximately 3300 flax accessions from around the world that have been characterized using the Random Amplification of

Polymorphic DNA – Polymerase Chain Reaction (RAPD-PCR) technique (Diederichsen and Fu, 2008) and extensive field and greenhouse evaluation. There is a broad diversity of useful characters that could be used to improve flax cultivars, including wilt resistance. When the genetic variation and relatedness of U. S. and Canadian cultivars were tested using 54 cultivars and 84 RAPD markers, moderate variation was observed with regard to primers, polymorphisms and cultivars (Fu et al., 2003). While the genetic relatedness of Canadian cultivars was less compared to U. S. cultivars, cultivars from the two countries showed a high level of intermixing. When the cultivars were clustered based on similarities, Canadian wilt susceptible cultivar Novelty (released in 1910) and U. S. wilt resistant cultivar Bison were grouped together.

Characterization of 153 flax accessions from around the world for fusarium wilt, anthracnose and pasmo diseases in the greenhouse and field nurseries in Russia, indicated that accessions from North and South America and from East Asia had above average flax wilt resistance, while accessions from Europe and the Indian subcontinent were below average (Diederichsen et al., 2008). This work identified flax accessions suitable for cultivation in different regions of the world based on previous reports of resistant cultivars from one location becoming susceptible at another location (Kommedahl, et al., 1970). Disease severity varied from 0 to 100% for the 153 flax accessions, indicating the variation in wilt resistance among flax lines or cultivars (Diederichsen and Fu, 2008). Canadian cultivars AC McDuff, Dufferin, Noralta and AC Emerson are highly resistant to flax wilt and displayed no symptoms.

Resistance to flax wilt is considered to be polygenic, because of the failure to observe any major resistance genes and the lack of evidence supporting race specific or vertical (single gene) resistance (Kommedahl et al, 1970). However, despite speculation of the existence of an infinite number of races of *Fol,* Knowles and Houston (1955) concluded

that two major genes conferred wilt resistance in flax. These genes, Fu_A and Fu_B, were identified using phenotypic ratios with two specific clones (isolates) of the pathogen. They considered four phenotypic classes in accordance with a 1:4:4:7 ratio of genotypes in the F_2 generation, inherited from homozygous resistant and susceptible parents, to fit with 0%, 25%, 44% and 100% disease severity. In a later study, the high wilt resistance of Dakota 48-94 to a third clone, was attributed to another gene Fu_C, inherited independently of the other two, proposing three major genes along with several minor genes that confer wilt resistance in flax (Knowles et al., 1956).

Flax wilt resistance was proposed to be due to major genes by Spielmeyer et al. (1998b). They suggested the effect of major resistance genes might be masked, due to the presence of many pathogen races in the soil, segregation of many genes from genetically diverse parents, and difficulties in identifying resistance in early stages of breeding programmes.

The presence of major genes in the parents, Linola line CRZY8/RA91 and cv. Glenelg was demonstrated in a study of doubled haploid lines (Spielmeyer et al., 1998b). These were inoculated with pathogen isolates under glasshouse conditions and a simple pattern of inheritance was observed that was suggested to be governed by one or two dominant genes. A significant number of doubled haploid lines displayed more extreme symptoms than the resistant parent, suggesting the involvement of minor loci. In the absence of major resistance genes or in the presence of several races of the pathogen, these minor genes were expected to contribute to the resistance expressed by the host (Spielmeyer et al., 1998b). Two quantitative trait loci explaining 38% and 26% of the phenotypic variation were mapped to linkage groups 6 and 10 in a later study using the same DH population, which was in accordance with the phenotypic observation of two major genes (Spielmeyer et al., 1998a). Major gene expression can be observed with single isolate inoculation in the absence other soil microorganisms in these experiments, since the competition for pathogen

survival in soil and infection of host, was reduced under controlled environmental conditions in the glasshouse.

In chickpea, seven physiological races of *F. oxysporum* f. sp. *ciceri* (Foc) have been identified, and the resistance to Races 0 and 5 were observed to be due to single genes from different linkage groups, with a phenotypic ratio of 1:1 for both races in a recombinant inbred line population (Tekeoglu et al., 2000). In a later study, Gowda et al. (2009) reported resistant to Races Foc2 and Foc3 also by single genes, and that Foc 1, 3, 4 and 5 might all belong to the same gene cluster.

Observations from studies of vascular wilt of melon caused by four races of the pathogen *F. oxysporum* f. sp. *melonis* (Fom), provided insight into the conclusions of researchers on *Fol* resistance genes (Perchepied et al., 2005). Two independent genes, *Fom*-1 and *Fom*-2, confer resistance to Races 1 and 2 and both genes provide resistance to Race 0, while resistance to Race 1.2 was identified to be polygenic, with nine quantitative trait loci (QTL) conferring resistance. Thus, different genes and QTLs confer resistance against each race of the pathogen with different inheritance patterns.

Controlled experiments conducted in glasshouses, growth chambers or under *in vitro* conditions can reduce environmental effects and provide more accurate observation of resistance gene expression (Kroes et al., 1998). Controlled environment studies are especially beneficial to determine the interactions of flax with soil-borne fungi, such as *Fusarium* spp., other than *Fol*. A highly significant correlation between field and glasshouse experiments was demonstrated by Spielmeyer et al. (1998b), thus substantiating the validity of controlled environment experiments in the greenhouse or growth chamber as predictors for field outcome. This is beneficial to breeding programs for selection of resistant lines in the early stages of the program.

2.9 Heritability

Heritability compares the influence of genes and environment on the variation of a phenotype and determines how heritable a characteristic is, in comparison of progeny to parents at a particular time (Visscher et al., 2008). Heritability is mostly population dependent, since both the genetic factor and the effect of environment vary for each population, and can change over time. It is of two types: broad sense heritability (H^2) and narrow sense heritability (h^2). Broad sense heritability is the proportion of phenotypic variance due to total genetic variance, which includes additive, dominance and epistatic variance, while narrow sense heritability is the proportion of phenotypic variance due to additive genetic variance.

Heritability of resistance to fusarium wilts seem to differ based on the *formae speciales*, the pathotype, the crop and the cultivar. The heritability of resistance to two pathotypes of race 1.2 of *F. oxysporum* f. sp. *melonis*, causing fusarium wilt in four genotypes of melon varied from 48% to 59% (Chikh-Rouhou et al., 2011). They had detected epistatic interactions and the resistance was assumed to be polygenically inherited. However, they had also observed genotype x pathotype interactions, indicating race specific resistance, which contradicted this observation. This was explained by Perchepied and Pitrat (2004), who suggested that race specific responses can be observed with polygenic resistance to specific pathogens, though this can be observed under very refined conditions.

2.10 Summary

Flax or linseed is important both as an oilseed and fibre crop. The two types are morphologically different and are predominantly grown in different regions, with Canada leading the world in flaxseed production. Breeding programs are designed to incorporate agronomic characters of economic interest into a new cultivar. Breeding for improved

agronomic characteristics in flax is challenging as it has plateaued for many traits, being an early domesticated, self-pollinated plant with a comparatively small diploid genome. Recombinant inbred line populations are developed by crossing two cultivars with desirable characteristics and making single seed selections for up to eight or nine generations to obtain a homozygous population and are used to incorporate traits of interest.

One prevalent disease of flax is wilt, caused by *Fusarium oxysporum* f. sp. *lini*, a facultative saprophyte that enters and colonizes the xylem, thus obstructing water movement. Disease severity depends on the resistance of the cultivar, virulence or aggressiveness of the isolate and the environment.

The best control method for flax wilt is the development and cultivation of resistant cultivars, along with integrated control methods such as crop rotation and fungicide treatment, to minimize selection pressure. Major gene resistance has been suggested against the disease, under controlled conditions. However, multigene resistance was observed under field conditions. Major gene expression is easier to detect in controlled environment tests, without the influence of environmental conditions or soil microflora. Observations under controlled conditions can be used as indicators of field performance of resistance against *Fol*.

Next generation sequencing has enabled rapid, low-cost sequencing of genomes, and is useful in conducting QTL mapping to identify the genes and QTLs associated with the expression of a trait. Since the race structure of the *Fol* pathogen is unknown, identification of the specific genes conferring resistance and their location in the genome is important in the development of wilt resistant flax cultivars and could contribute to marker assisted selection in breeding programs.

Chapter 3: Evaluation of Resistance of Four Flax Cultivars to Seventeen *Fusarium oxysporum* f. sp *lini* Isolates Under Controlled Conditions

3.1 Abstract

Seventeen *Fusarium oxysporum* f. sp. *lini* (*Fol*) isolates were used to inoculate four flax cultivars: Bison, Novelty, Aurore and Oliver, to identify three isolates to use to screen a RIL population derived from a cross of Aurore and Oliver, for resistance for flax wilt. Vermiculite supplemented with Murashige and Skoog (MS) medium as the growth medium, with growth chamber temperature at 23°C and moist conditions maintained with closed trays, were selected for optimal disease development, during the four-week period of disease assessment. A disease grading scale ranging from 0 to 9 based on yellowing, wilting and plant height reduction was used to assess the amount of disease, and the height of each plant was measured each week. Isolates differed in area under the disease progress curve (AUDPC) and disease severity, and were categorized into three groups based on aggressiveness. Plant height was inversely correlated with AUDPC, indicating the negative effect of flax wilt on plant growth. Based on four criteria and using AUDPC, three isolates (Isolates 65, 81 and 131) were selected to screen the recombinant inbred line (RIL) population.

3.2 Introduction

Flax (*Linum usitatissimum* L.) is grown either for fibre, which is used to produce linen and paper, or flaxseed (i.e. linseed), consumed as a whole seed or from which to extract edible oil rich in α-linolenic acid or used in high quality linoleum flooring or paints (Amin & Thakur, 2014). The first oilseed crop to be widely grown in Western Canada, 40% of the world's linseed is produced in Canada, of which approximately 70% comes from Saskatchewan (Duguid, 2010; Rowland, 2014).

Fusarium oxysporum f. sp. *lini* (Bolley) Snyder & Hanssen, causing flax wilt, is an important soil-borne pathogen of flax. It is a facultative saprophyte, producing three types of spores, microspores, macrospores and chlamydospores, the latter being a dormant spore with thick cell walls enabling the *Fol* pathogen to survive a long time in the absence of a host (Kommedahl et al., 1970). Houston and Knowles (1949) reported an outbreak of flax wilt in a field where flax was grown after 50 years, providing an indication of the long-term survival of the pathogen. This necessitates the development of resistant flax cultivars since it is impossible to economically and effectively eradicate a soil-borne saprophyte.

The existence of races of *Fol* has been debated for many years. Early studies have reported the occurrence of many races, which differ in terms of physiological characteristics and pathogenicity (Kommedahl et al., 1970; Flor, 1953). In the experiments by Broadfoot and Borlaug, antagonism among races was observed supporting this theory (as cited in Flor, 1953). However, Kommedahl et al. (1970) speculated they might belong to a single race, with minor genetic variation among isolates erroneously identified as races. Knowles et al. (1956) reported race-specific resistance in *Fol* under glass-house conditions using sterile growth medium, indicating the existence of a few races, which Spielmeyer et al. (1998b) theorized was due to a lack of competition among isolates and increased infection under optimal conditions. In contrast, many other *formae speciales* of *F.*

oxysporum, have been reported to have only a limited number of races; e.g. eight physiological races in *F. oxysporum* f. sp. *ciceris* cause of wilt in chickpea and three races each in *F. oxysporum* f. sp. *vasinfectum* causing fusarium wilt of cotton and *F. oxysporum* f. sp. *cubense* causing fusarium wilt of banana (Jiménez-Fernández et al., 2013; Assigbetse et al., 1994; Li et al., 2013).

Multiple factors affecting the inheritance of flax wilt resistance have been suggested and extensive research has identified resistant germplasm. While cultivars completely immune to flax wilt have not been reported, there are resistant cultivars such as AC Linora, AC Emerson, AC McDuff, Flanders and Hanley. All recommended North American flax cultivars have at least moderate resistance to flax wilt (Rashid, 2003). Shifts in the pathogen population can result in resistance break down in flax cultivars (Kommendahl et al., 1970; Flor, 1953). The cultivar Bison is an exception in this regard as it has retained flax wilt resistance since its release in 1925 (Kommendahl et al., 1970; Arny, 1943). However, Bison and the wilt susceptible cultivar Novelty, were grouped together, based on agronomic characteristics, in a study conducted to identify the genetic relationships among North American flax cultivars (Fu et al., 2003).

A flax wilt phenotyping method was modified to test flax lines, under controlled environmental conditions. Seventeen isolates collected from flax fields in Manitoba and Saskatchewan were used in this study and their aggressiveness/ virulence on four flax cultivars was tested; North American cultivars, Novelty, wilt susceptible and Bison, wilt resistant, and French cultivars Aurore, wilt resistant and Oliver, wilt susceptible. Disease assessment was carried out for four weeks, post inoculation, and the area under disease progress curve (AUDPC) was calculated to identify the similarities among the isolates and identify two pathogen isolates to use in a later study (Chapter 4).

3.3 Methodology

The protocol to screen flax cultivars for flax wilt in the growth chamber was modified to overcome the issues encountered with respect to plant growth and vigour. The modified protocol was used to carry out the preliminary disease assessment using 17 isolates.

3.3.1 Plant Material

The parents of aFrench RIL population: the spring, fibre type, Aurore (Argos x Nynke, resistant to flax wilt) and the winter, linseed type, Oliver (Hiver15 x Forget15, susceptible to flax wilt) were used as test cultivars. The American flax cultivar Bison, which is resistant to flax wilt was obtained from the AAFC, Morden flax breeding program. The Canadian cultivar Novelty, reported to be susceptible to *Fol* was obtained from the Flax Breeding Program at the Crop Development Center, Saskatoon. These cultivars were used as resistant and susceptible checks.

3.3.2 Growth Media

Growth medium was prepared by supplementing 15 L of vermiculite with 8 L of 0.4 g/L Murashige and Skoog basal medium (Sigma-Aldrich. Inc., St. Louis, MO) and autoclaving at 121°C, for 15 min. Ten cm square pots were filled with the autoclaved mixture and eight pots were placed on plastic saucers in a tray, to prevent mixing of the water leaching from the pots. All trays were covered with transparent lids to maintain humidity and to prevent drying of the vermiculite.

Nine seeds were sown in a pot at an approximate depth of 1.5 cm and plants were thinned to seven seedlings, one week after seeding. The trays were placed in the growth chamber with a 16 h day at 23°C temperature and 8 h night at 18°C. Plants were watered

daily with 50 ml of water and 50 ml of 0.4 g/L of MS medium was added to each pot three weeks after sowing, to provide sufficient nutrients for the plants.

One replicate of each isolate x cultivar combination was placed in each of two growth chambers, in a randomized complete block design (RCBD). Each replicate was sown and inoculated on subsequent days, and the experiment was repeated once. All isolate x cultivar (or control) combinations were randomized within each replicate.

3.3.3 Inoculum Preparation

Seventeen *Fol* isolates were provided by Dr. Khalid Rashid (AAFC, Morden, Manitoba), collected from different fields in Manitoba or Saskatchewan, Canada. They were sub-cultured on Potato Dextrose Agar (PDA) medium and refrigerated. Fresh cultures of each isolate were prepared on PDA seven days prior to inoculum preparation to obtain optimal fungal growth.

A sterile 10 mm cork borer was used to cut agar plugs from the growing edge of a colony and five plugs were transferred to 125 ml of autoclaved Czapek Dox medium in a 250 ml Erlenmeyer flask. Three flasks were prepared per isolate at a time. These were incubated at approximately 22°C on the shaker at 100 rpm at 16:8 hour day/ night. After seven days, the spore suspension was filtered through sterile cheese cloth into a sterile flask and the filtrate of the three flasks combined. A haemocytometer was used to quantify the inoculum and the spore concentration was adjusted to 10^6 spores/ml using sterile distilled water. Half of the spore suspension was refrigerated to inoculate Replicate 2 the next day. A fresh batch of spore suspension was prepared for each experiment to maintain high germination and virulence of the spores. The second replicate was inoculated with the refrigerated spore suspension, after letting it reach room temperature.

3.3.4 Inoculation and Disease Grading

Seven day old seedlings of Aurore, Oliver, Bison and Novelty were inoculated with 15 ml of spore suspension of each isolate. Spore suspension was added to the surface of the vermiculite so that it was distributed throughout the pot. In each replicate of each experiment all four cultivars had a control pot that was inoculated with 15 ml of sterile distilled water, which was used to prepare the spore suspension. Lids were replaced after inoculation.

Disease assessment was conducted, using a 0-9 disease grading scale (Table 3.1, Figure 3.1) beginning seven days after inoculation (dai), and every seven days thereafter until 28 dai for each pot. The height of all the plants in a pot was measured. Average plant height was calculated for each pot as a percentage of the average height of the control.

Table 3.1 Flax wilt disease assessment scale, used in growth chamber and field experiments. Disease was assessed as the percentage of plant tissue showing the disease symptoms in a pot or row.

Grade	Symptoms
0	No symptoms, the most vigorous
1	Vigorous, yellowing on 0–5% of the plants
2	Vigorous, yellowing on 5–10% of plants
3	Slight reduction in vigour, yellowing on 10–20% of plants, slight reduction in height or branching. No severely wilted or dead plants
4	Moderate vigour, yellowing on 20–40% of plants, moderate reduction in height or branching and/or <1% severely wilted or dead plants
5	Moderate vigour, yellowing on 40–60% of plants, moderate reduction in height or branching and/or 1–10% severely wilted or dead plants
6	Poor vigour, yellowing on >60% of plants, moderate reduction in height or branching and/or 10–30% severely wilted or dead plants
7	Poor vigour, yellowing, severe reduction in height or branching and/or 30–60% severely wilted or dead plants
8	Very poor vigour, severe reduction in height or branching and/or 60–90% severely wilted or dead plants
9	All plants severely wilted or dead

Figure 3.1. Plants at different stages of disease development, rated 0, 3, 6 and 9 on the disease assessment scale (Table 3.1) at 28 days after inoculation with isolates of *Fusarium oxysporum* f. sp. *lini*.

All plants in a pot were considered one unit and assigned a rating based on the disease severity of the seven plants. The four disease scores were used to calculate the AUDPC using the following formula:-

$$\text{AUDPC} = \sum_{i=1}^{n-1} \{(X_{i+1} + X_i) / 2) (T_{i+1} - T_i)\}$$

where,

X_i is disease severity at the i^{th} observation,

T_i is the time of the first disease rating, and

n is the total number of scoring dates in the trial.

The average height of the plants in each pot was taken as one replicate. Height was expressed as a percentage of the control, for this study.

3.3.5 Statistical Analysis

All phenotypic data were analyzed using SAS 9.3 statistical software (SAS Institute Inc., Cary, NC, USA). The data significantly deviated from normality and homogeneity of variance though normality was achieved with residuals only for disease severity at 28 dai. Therefore, the data was fit to a generalized linear mixed model (Proc GLIMMIX) with a

Poisson distribution, with replication within experiments as a random effect. Outliers were removed to reduce the standard deviation of the data set. The Tukey test was used to calculate multiple pairwise comparisons among the cultivars, isolates and their interactions, using least square means. Principle component analysis was performed to group the isolates based on aggressiveness. Pearson's correlation coefficients were calculated for disease severity at 21 and 28 dai and plant height (expressed as a percent of the control) at 28 dai and AUDPC.

3.4 Results

3.4.1 Optimization of the protocol

The modifications of the protocol for flax wilt screening, from the original protocol of Dr. K. Rashid included the addition of 50 ml of 0.4 g/L MS medium to each pot three weeks after seeding to maintain the continuous supply of nutrients, and covering of the trays with a lid to retain moisture, since the moisture evaporated rapidly due to the air flow in the growth chamber. Additionally, this increased the temperature within a tray by 1-2 °C, providing optimal daytime temperature for the pathogen. Also, the covers helped to reduce the thrip (Order Thysanoptera) damage to the plants.

3.4.2 Preliminary Disease Assessment

Isolate 65 was collected from Indian Head, SK, Isolate 66 from Saskatoon, SK, Isolate 159 from Oakville, MB and the remaining 14 isolates from Treherne, MB. The symptoms varied among isolates and cultivar combinations. Initial symptoms were yellowing of the leaves (chlorosis), leading to browning of the leaves (necrosis) and completely wilted plants, and some plants had the characteristic feature of bending of the apex to form a shepherd's crook (Figure 3.2). However, there were exceptions, where plant necrosis occurred without initial yellowing, with plant wilting and browning started from the apex, especially with aggressive isolates and susceptible cultivars. When the plants were pulled

from the vermiculite, the root system of plants that displayed severe and early onset of disease were grey and less developed, compared to plants that had less severe disease symptoms or later disease onset (Figure 3.3)

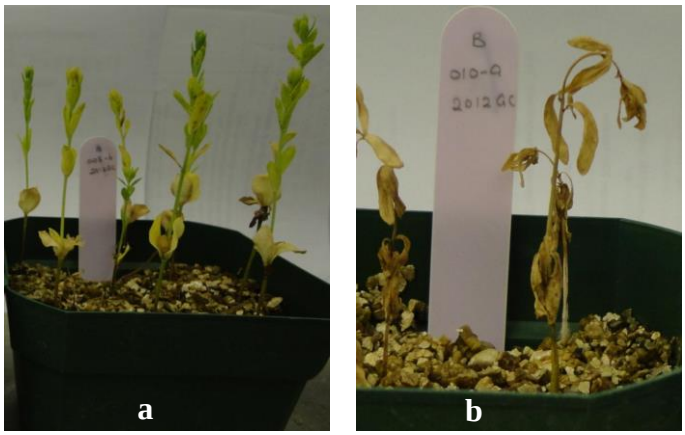


Figure 3.2. Progression of symptoms of *Fusarium oxysporum* f. sp. *lini* infected flax plants: a) yellowing; b) browning, wilting and "shepherd's crook".

i ii i ii

(a) (b)

Figure 3.3. a) Roots system of flax plants, 28 days after inoculation with *Fusarium oxysporum* f. sp. *lini* isolates: (i) rating of 2 (0-9 scale), well developed and asymptomatic; and (ii) rating of 9, less developed and grey, and b) the same roots observed under the microscope at x400 magnification.

The Generalized Linear Mixed Model, which accommodates datasets with correlations, heterogeneous variation or non-normal distribution, was used to model the data and examine the variation since the data deviated from the expected normal distribution and homogenous variation. Cultivar by isolate interactions indicated a wide range of disease severity at 21 and 28 dai and AUDPC (Table 3.2).

Table 3.2. F values of Type 3 tests of fixed effects of the GLIMMIX procedure for disease severity (DS) and area under the disease progress curve (AUDPC) at 21 and 28 dai with 4 cultivars and 17 isolates and coefficient of variation (CV) for DS and AUDPC.

	DS at 21 dai	DS at 28 dai	AUDPC
Isolate	55.17***	54.15***	85.75***
Cultivars	66.87***	94.22***	117.48***
Isolate*Cultivars	4.28***	4.75***	5.87***
CV (%)	22.4	16.64	18.53

*** P <0.001

Isolates, cultivars and isolate x cultivar interactions were all significant for disease severity at 21 and 28 dai and AUDPC, although the variation was higher for isolate and cultivar than for the interactions. This implies that most of the variation in disease reaction was due to the different isolates and cultivars with little contribution from the interaction of the variables. However, a significant isolate by cultivar interaction can be an indication of race-specific resistance.

Further comparisons with the Tukey's test indicated there was no significant difference between Aurore and Bison for disease severity at 21 dai or AUDPC, while they were significantly different for disease severity at 28 dai. All other comparisons between the cultivars were significant (Appendix 1).

Initially, differential disease development of an isolate on the two parents and the checks were tested and all the isolates showed significant interactions with the cultivars at the 5% significance level. Pairwise comparisons between the isolates for the three variables, showed several isolates that were not significantly different from one another. Principle component analysis identified three groups based on isolate aggressiveness towards the four cultivars tested (Figure 3.4). This combined with non-significant differences among isolates based on the level of aggressiveness given by least square means, was used to divide the isolates into three groups (Table 3.3).

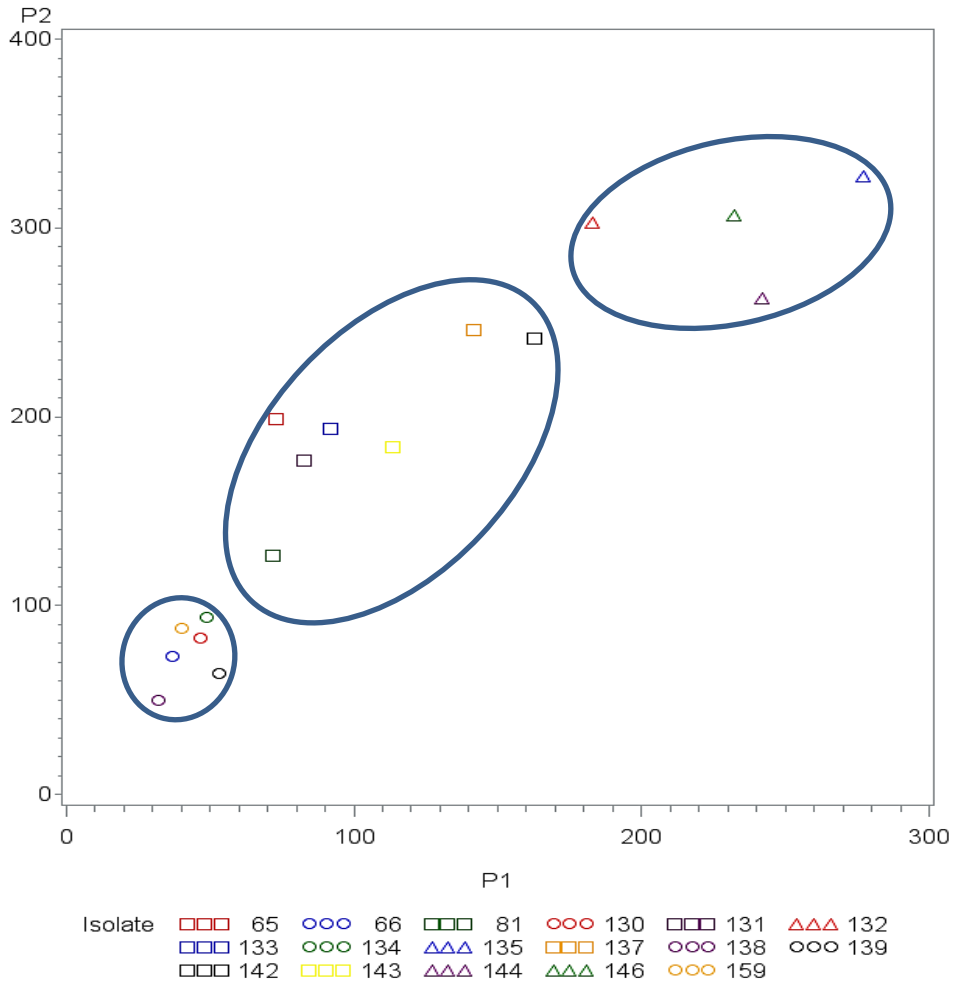


Figure 3.4 Principle component analysis for aggressiveness of 17 *Fusarium oxysporum* f. sp. *lini* isolates based on disease severity at 21 and 28 days after inoculation and area under disease progress curve on flax wilt resistant cultivars Bison and Aurore, and flax wilt susceptible cultivars Novelty and Oliver. Isolates are grouped as (○) weak, (□) moderate and (△) extreme.

Table 3.3. Aggressiveness of 17 isolates of *Fusarium oxysporum* f. sp. *lini* based on results from preliminary disease screening with cultivars Bison, Novelty, Aurore and Oliver under controlled conditions. The categorization was based on results from principle component analysis and Tukey's pairwise comparisons of the 17 isolates.

Extremely aggressive	Moderately aggressive	Weak
132	65	66
135	81	130
144	131	134
146	133	138
	137	139
	142	159
	143	

The AUDPC of the 17 isolates varied greatly among cultivars. Disease severity and AUDPC was always greatest for Oliver. Bison often displayed the lowest severity scores, under controlled conditions. All 17 isolates behaved in the same manner over the four cultivars, with aggressive isolates causing severe disease on all four cultivars and vice versa. (Figure 3.5)

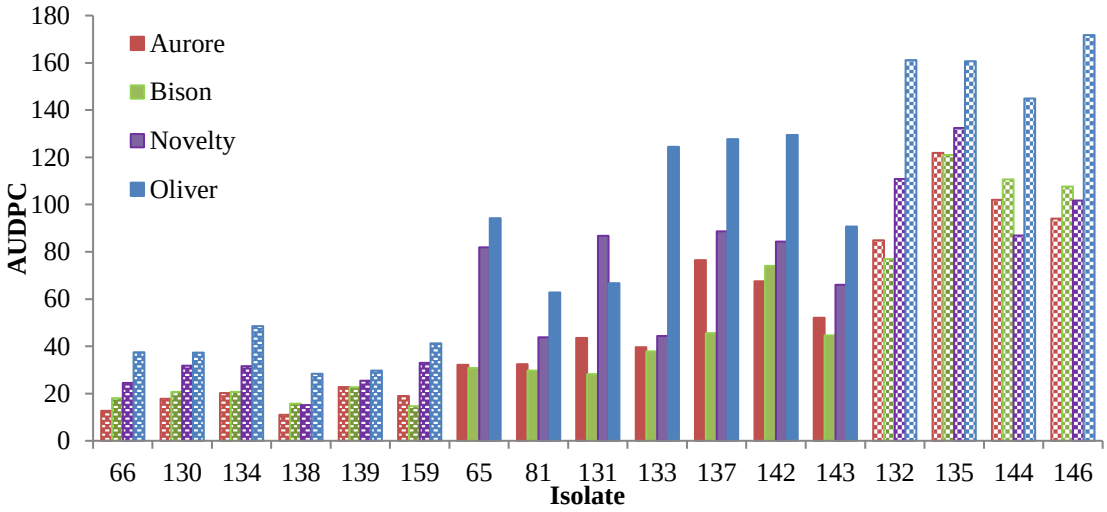
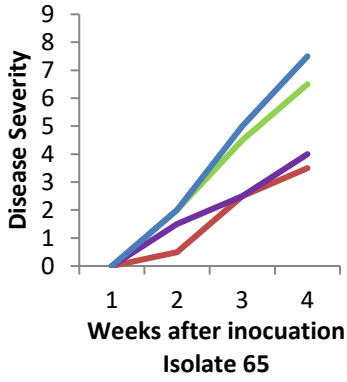
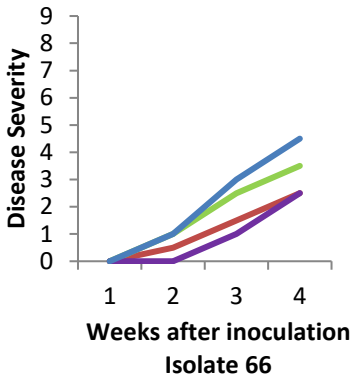
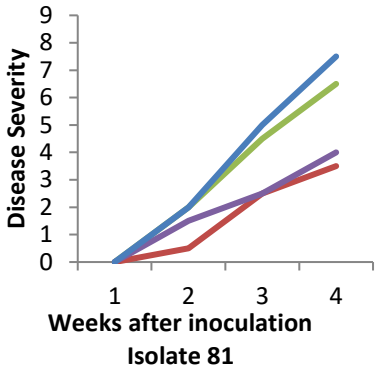


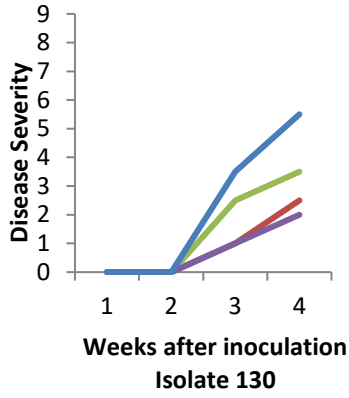
Figure 3.5 Area under disease progress curve (AUDPC) of the 17 isolates with each cultivar. The isolates were grouped as weakly (▩), moderately (■) and extremely (▩) aggressive based on principle component analysis and Tukey's pairwise comparisons.

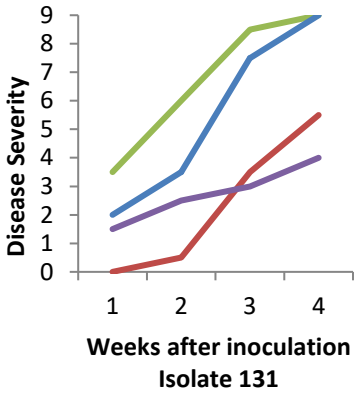
Disease progression over the four-week period was observed to differ among the isolates and the cultivars. All four cultivars showed severe disease symptoms by 28 dai with extremely aggressive isolates i.e. isolates 132, 135, 144 and 146, while low disease severity scores were reported with weak isolates. Better separation of resistant and susceptible cultivars and gradual progression of disease over the 28-day assessment period, was observed with moderately aggressive isolates: Isolates 65, 81 and 131 (Figure 3.6).

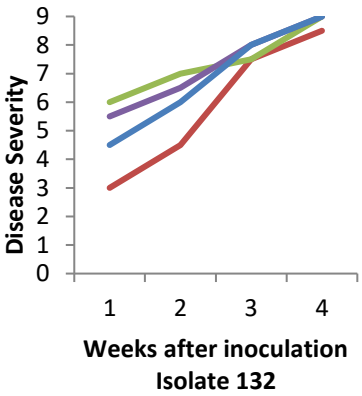

Disease Severity
0 1 2 3 4 5 6 7 8 9
1 2 3 4
Weeks after inocuation
Isolate 65

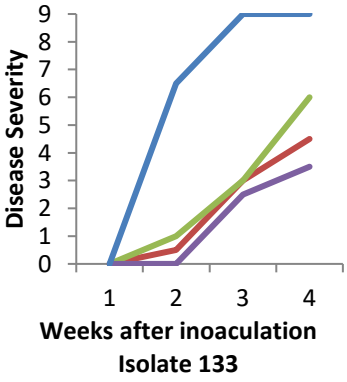

Disease Severity
0 1 2 3 4 5 6 7 8 9
1 2 3 4
Weeks after inoculation
Isolate 66

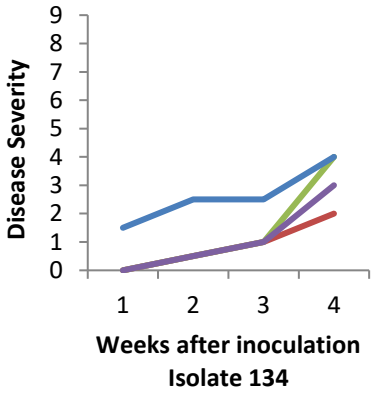

Disease Severity
0 1 2 3 4 5 6 7 8 9
1 2 3 4
Weeks after inoculation
Isolate 81

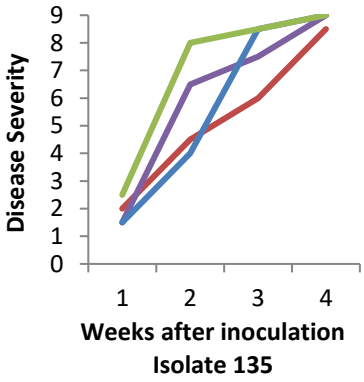

Disease Severity
0 1 2 3 4 5 6 7 8 9
1 2 3 4
Weeks after inoculation
Isolate 130

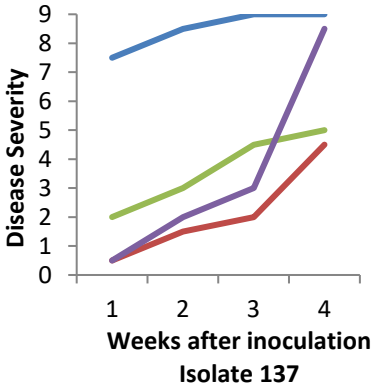

Disease Severity
0 1 2 3 4 5 6 7 8 9
1 2 3 4
Weeks after inoculation
Isolate 131

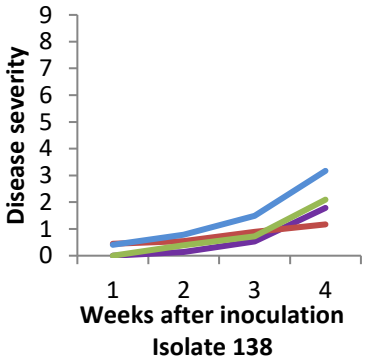

Disease Severity
0 1 2 3 4 5 6 7 8 9
1 2 3 4
Weeks after inoculation
Isolate 132

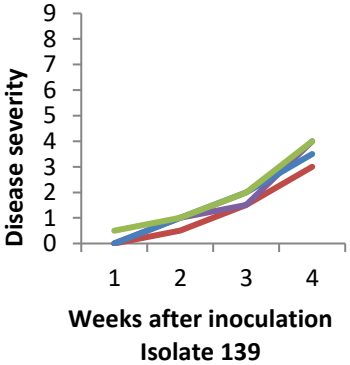

Disease Severity
0 1 2 3 4 5 6 7 8 9
1 2 3 4
Weeks after inoaculation
Isolate 133


Disease Severity
0 1 2 3 4 5 6 7 8 9
1 2 3 4
Weeks after inoculation
Isolate 134


Disease Severity
0 1 2 3 4 5 6 7 8 9
1 2 3 4
Weeks after inoculation
Isolate 135


Disease Severity
0 1 2 3 4 5 6 7 8 9
1 2 3 4
Weeks after inoculation
Isolate 137


Disease severity
0 1 2 3 4 5 6 7 8 9
1 2 3 4
Weeks after inoculation
Isolate 138


Disease severity
0 1 2 3 4 5 6 7 8 9
1 2 3 4
Weeks after inoculation
Isolate 139

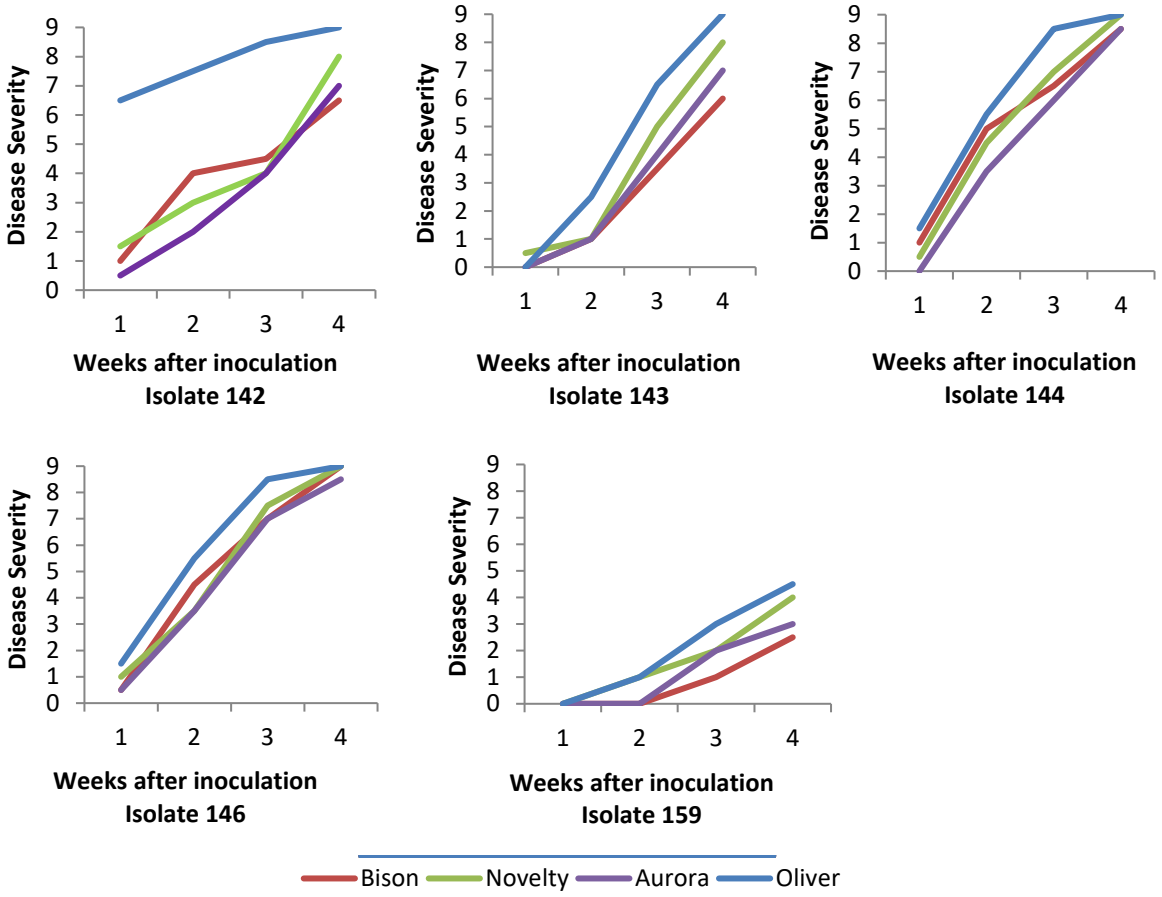


Figure 3.6 Flax wilt severity of flax cultivars Bison and Aurore (resistant), and Novelty and Oliver (susceptible), over four weeks, after inoculation.

Significant correlations were observed between AUDPC and disease severity at 28 dai, while moderate yet significant, inverse correlations were observed for height (expressed as a percent control) at 28 dai with AUDPC and disease severity at 21and 28 dai (Table 3.4).

Table 3.4. Pearson's correlation coefficients for disease severity (DS) at 21 and 28 dai, height as a percentage of the control at 28 dai and area under the disease progress curve (AUDPC) for 17 isolates of *Fusarium oxysporum* f. sp. *lini* on flax cultivars Bison, Novelty, Aurore and Oliver.

	DS at 21 dai	**DS at 28 dai**	**AUDPC**
DS at 28 dai	0.90***		
AUDPC	0.97***	0.91***	
percent control height	-0.57***	-0.55***	-0.56***

*** P <0.001

3.5 Discussion

The objective of this experiment was to develop a method that could be used to successfully screen a RIL population under controlled conditions. The flax wilt screening protocol of Dr. K. Rashid (unpublished) was optimized to suit the growth chamber conditions. In the protocol, vermiculite was used as the growth medium since it provided an aerated medium where roots developed well during the limited period of disease assessment, and provided good drainage. Vermiculite was also able to hold more water than soil, thus ensuring that the plants were not wilting due to drying, an important aspect of this study. Additionally, it has been found that vermiculite was able to develop more mycorrhizal inocula, indicating it provides a better medium for fungal growth (Martin, 2001). Murashige and Skoog medium was added to provide essential elements, lacking in vermiculite. Autoclaving removed any competing pathogens that might have existed, thereby providing the optimal growth medium for pathogen growth.

During the first few trials, a significant amount of thrip damage was observed on the flax seedlings, and the vermiculite dried out rapidly due to air movement caused by exhaust fans used to keep the room temperature constant. Therefore, trays were covered with transparent lids, even though it created 'micro-environments' within the trays. The lids increased the temperature within a tray, improving conditions for the pathogen.

Czapek-Dox was used for preparation of inoculum since it is a minimal medium and promoted sporulation of *Fol*, which ensured a sufficient amount of spores would be present. Of the 17 isolates, only three were collected from locations other than Treherne, MB; Isolate 65 from Indian Head, SK, Isolate 66 from Saskatoon, SK and Isolate 159 from Oakville, MB.

Infected plants showed a range of disease symptoms. The most obvious was significant browning and wilting of susceptible plants. Chlorosis was observed at various levels at

disease onset, which soon developed into necrosis (indicated by browning). In a study of the *Fol* infection process, it was observed that the pathogen entered the plant through lateral roots or root hairs followed by intercellular and intracellular establishment in the cortex (Kroes et al., 1998). This resulted in rapid activation of defense mechanisms in the plant, such as production of new layers of calyptra and formation of appositions around the penetration hyphae. Uncontained pathogen hyphae eventually grew into the vascular system of the pathogen, after exhausting the nutrients from the affected cortex cells. This led to wilting of the plant. It was observed that while the defense mechanisms between resistant and susceptible cultivars were similar, more prominent appositions and intercellular phenolic compound contents were present in the resistant cultivar. Therefore, the difference in *Fol* resistance or susceptibility in flax depended on activation of defense reactions with infection of *Fol*.

Isolates, cultivars and their interactions were all significant for the three variables tested. Significant isolate by cultivar interactions suggested race specific resistance, though no races have been identified for *Fol*. However, since the interaction effect is smaller than either the isolate or cultivar effects, this may be described as a "minor-gene-for minor-gene" model effect. The term was described by Marcel et al. (2008), explaining the partial resistance to leaf rust (*Puccinia hordei*) in barley. Rogers and Stevens (2010) observed similar isolate by cultivar interactions, with isolates of *Alternaria, dauci* (cause of Alternaria leaf blight) in carrot, though races of *A. dauci* have not been described. They reported possible minor-gene interactions between isolates and cultivars observed under a controlled environment. Their study also showed the importance of controlled conditions for the separation of isolates, based on their aggressiveness.

When the two experiments were analyzed, a high coefficient of variation (CV) was observed, which explains the relative dispersion of a variable. It is calculated by dividing

standard deviation by the mean, and is independent of a measurement unit. Use of a mixture of isolates as the inoculation, with increased number of replications has been suggested to reduce CVs (Sounigo et al., 2003). However, in this analysis, the extreme values in the data, creating the high variation was removed.

The level of aggressiveness of the four cultivars was observed to differ among the isolates. Based on least square means and principle components, isolates were grouped into three categories; weakly, moderately and extremely aggressive. There were six weakly aggressive isolates, whereas Isolates 132, 135, 144 and 146 were extremely aggressive. However, with only four cultivars used for disease assessment, for which gene for resistance are unknown the isolates could not be characterized into races, however the significant isolate by cultivar interaction suggested a gene-for-gene interaction, the basis of race characterization. Race structure studies of *Fol* have not been conducted on Western Canadian isolates, although two isolates from Morden were included in a race structure study conducted in the Netherlands, using 25 isolates collected from around the world (Kroes et al., 1997). Researchers were not able to identify distinct races among the isolates, concluding that races based on minor gene differences might occur, especially distinguishable between *Fol* originating from North America and Europe.

Bison was the most resistant and Oliver the most susceptible of the cultivars, though there was no significant difference between Bison and Aurore, without considering disease response to individual isolates. Aurore and Oliver were significantly different, indicating the RIL population generated would be segregating for *Fol* resistance, which would be useful in identifying resistant quantitative trait loci (QTLs) in the latter part of the study.

Finally, Pearson's correlation coefficient indicated a high, significant correlation between disease severity at 21 and 28 dai and AUDPC. All three variables demonstrated a strong inverse correlation with plant height, indicating that *Fol* infection affects plant

growth. Since plant height and yield are correlated, reduced plant height can result in reduced yield (Zhang, 2013).

Based on the results of this study, three isolates were selected to screen a recombinant inbred line population for resistance to flax wilt (Chapter 4). The same protocol optimized in this study was used in disease phenotyping.

Chapter 4: Wilt Reaction of a Recombinant Inbred Line Population Derived from a Cross of Aurore and Oliver

4.1 Abstract

Three *Fusarium oxysporum* f. sp. *lini* (*Fol*) isolates (Isolates 65, 81 and 131) were selected to phenotype a recombinant inbred line (RIL) population of a cross between cultivars Aurore (wilt resistant) and Oliver (wilt susceptible), under controlled environment, which was in accordance with four selection criteria. All three isolates had a similar pattern, a gradual increase in disease severity (DS) and the area under the disease progress curve (AUDPC) at 21 and 28 days after inoculation (dai). Height was inversely correlated with both variables for Isolates 65 and 81, but not for Isolate 131. There were very few RILs with resistance greater than the resistant parent, although the resistance of the lines was not always consistent among the three isolates. Observations were similar in the field wilt nursery experiments conducted at three site years with no separation of resistant and susceptible lines. Lines more resistant than Aurore or more susceptible than Oliver were identified in the wilt nursery studies, although they were not mean values since it was not a replicated experimental design. There was a significant difference among site years for the variables tested, indicating a strong environmental effect. Correlations between controlled environment trials and wilt nursery experiments were moderate, but always significant. Hence, single isolate phenotyping in controlled environment can be used as predictors for wilt resistance of flax cultivars in field conditions. Moderate, correlations of RIL phenotypes were observed between Canadian and French wilt nurseries.

4.2 Introduction

Cultivar development requires efficient selection of disease resistant germplasm. This leads to the necessity of developing effective and economical methods to screen plants for disease resistance, especially with soil-borne diseases such as flax wilt. This disease is caused by one of the most ubiquitous fungal species in the soil, *Fusarium oxysporum* f. sp. *lini* (Bolley) Snyder & Hanssen. This species is a saprophyte, producing three types of spores: microspores, macrospores and chlamydospores, the latter a dormant spore with thick cell walls enabling the pathogen to survive a long time in the absence of a host (Kommedahl *et al.*, 1970). Houston and Knowles (1949) reported an outbreak of flax wilt in a field where flax was grown after 50 years, indicating the long-term survival of the pathogen. This requires the development of resistant flax cultivars, since it is challenging to eradicate a soil-borne saprophyte.

When developing a disease resistant plant cultivar, it is important to consider the virulence of the pathogen strains/ races/ pathotypes in a specific region. Therefore, during variety development, it is essential to test for resistance effective against the prevalent pathogen strains in a region, especially for major gene resistance (Spielmeyer *et al.*, 1998a). In early experiments, cultivars identified to be resistant at one location were observed to be susceptible at another location, indicating the presence of different pathotypes among locations. In 1954, 59 wilt resistant cultivars, developed over 34 years in Minnesota, were re-tested for flax wilt. It was observed that 62% of the cultivars from the 1920's were susceptible by 1954, whereas only 4% of the 1930's – 1940 cultivars were susceptible. This indicated a shift in *Fol* pathotypes at a specific location and loss of resistance towards old pathotypes (Kommedahl *et al.*, 1970).

In a more recent study in the Netherlands isolates and cultivars from different countries were used to demonstrate the potential existence of races of *Fol* with minor gene differences

(Kroes *et al*., 1997). This is partially in accordance with a similar study, in which two independent genes were concluded to confer resistance in a controlled environment (Spielmeyer *et al,* 1998b). The expression of minor genes was determined to govern the resistance response in the presence of multiple races of a pathogen and varied environmental effects in fields or heterogeneity of genes segregating from diverse parents (Spielmeyer *et al,* 1998b).

In the experiment by Kroes *et al*. (1997), American linseed cultivar Bison was included, and was observed to be highly resistant 90 years after it was first developed in North Dakota. Using cultivars Bison and Novelty as resistant and susceptible checks, the present study was designed to compare the phenotypic variation in a recombinant inbred line (RIL) population under a controlled environment and under field conditions in wilt nurseries. Also, the predictability of field performance of a specific cultivar, using controlled environment phenotyping data was analyzed.

4.3 Methodology

4.3.1 RIL Phenotyping in the Growth Chamber

4.3.1.1 Plant Material

A RIL population consisting of 200 lines was developed from a cross of French flax cultivars: Aurore (moderately resistant [MR] to flax wilt; spring, fibre type) and Oliver (susceptible [S] to flax wilt; winter, linseed type) were provided for this study along with the parents. First crossed in 2000, F_1 seeds were grown in a south hemisphere nursery in the winter of 2000/2001 and 300 seeds were harvested from one F_1 plant. These were planted in May 2001 in a field nursery in France and 200 F_2 plants were harvested. Approximately 80 seeds were harvested per plant in the F_3 generation. One F_3 plant in the middle of the row was harvested to give the F_4 generation; this was repeated every year, up to the F_8 generation. The final population that was provided was from bulked seed derived

from a single F_8 plant generation. These seeds were increased in the growth chamber and seeds from each single plant increase was used in RIL phenotyping in the growth chamber.

North American flax cultivars Bison, which is resistant (R) and Novelty, which is susceptible (S) to *Fol*, were obtained from the Crop Development Center's Flax Breeding Program, and were used as checks in the experiment.

A subset of 160 of the 200 RILs was used for the controlled environment phenotyping due to space limitations and availability of seeds, since some of the RILs produced fewer seeds than required in the growth chamber seed increase. Parents of the RILs, Aurore and Oliver were included along with the checks Bison and Novelty.

Autoclaved vermiculite supplemented with MS medium was added to 21 trays with eight pots per tray. After planting these were placed in the growth chamber with a 16 h day at 23°C temperature and 8 h night at 18°C. Nine seeds were sown per pot and thinned to seven plants per pot seven days after seeding. To ensure the medium was not depleted of necessary nutrients, 50 ml of 0.4 g/L of sterilized MS medium was added to each pot three weeks after sowing. Plants were watered daily as required and the trays were covered with lids to preserve the moisture.

The RILs, parents and checks were randomized within a chamber, using the command *RAND* in Microsoft Excel. The experiment had two randomized replications; each in a different growth chamber, and the experiment was repeated once. Within each replication, controls of the resistant and susceptible checks and parents inoculated with sterile distilled water, were included. Seeding and inoculation in the two chambers (replicates) were carried out over two consecutive days to accommodate the work-load.

4.3.1.2 Inoculum Preparation

Isolates 65, 81 and 131 were selected based on the preliminary disease screening (Chapter 3) to conduct the RIL phenotyping. All three isolates were sub-cultured on PDA medium, seven days prior to inoculum preparation to obtain optimal fungal growth.

Ten Erlenmeyer flasks containing 125 ml of Czapek Dox medium were inoculated with five agar plugs cut with a sterile 10 mm cork borer from the growing edge of the culture. These were incubated at 25°C on a shaker at 100 rpm at 16:8 hour day/ night. The spore suspension was filtered through sterile cheese-cloth after seven days of incubation and the filtrate of the ten flasks was combined. The concentration of the spore suspension was determined using a haemocytometer. Finally, the spore concentration was adjusted to 10^6 spores/ml using sterile distilled water. A new batch of spores was prepared for each experiment, and half of the suspension was refrigerated to inoculate the second replicate the following day.

4.3.1.3 Inoculation and Disease Grading

Seven day old seedlings of the 160 RILs, parents and checks were inoculated with 15 ml of the *Fol* spore suspension using a 5 ml pipette. The spore suspension was added to the surface of the vermiculite so that it was distributed throughout the pot. In each experiment, 15 ml of sterile distilled water, which was used to prepare the spore suspension, was added to control pots of Novelty, Bison, Aurore and Oliver. After inoculation, the trays were covered again with the lids, which increased the temperature by 1-2°C, providing ideal conditions for the pathogen.

Disease severity of the seven plants in each pot was collectively assessed using the same grading scale as in Chapter 3 and the heights of the individual plants in the pot were also recorded, beginning seven days after inoculation (dai) to 28 dai, at seven day intervals. From the disease scores the AUDPC was calculated for each RIL at 21 and 28 dai.

4.3.2 RIL Phenotyping in Wilt Nurseries

4.3.2.1 Plant Material

Two hundred RILs, the parents and the cultivars Novelty and Bison, were evaluated in the wilt disease nurseries at Saskatoon, SK and Morden, MB in the Summer of 2013 and 2014 and assessed for flax wilt disease at three growth stages.

4.3.2.2 Experimental Design

The experiment was carried out in a Modified Augmented Design (MAD). The MAD uses uniformly distributed control plots in the center of whole plots and randomly distributed control subplots to assess the soil and environmental variability in the nursery, without replication of individual lines. Agrobase Generation II software was used to do the randomization and determine the experiment layout.

To fit into the wilt nursery space, plots were arranged in a 6 x 6 grid, with each whole plot containing seven subplots with control plots in the middle (Figure 4.1). Bison and Novelty were used as plot and sub-plot controls, while Aurore and Oliver were included as test plots along with the RILs. Bison (wilt resistant) was selected as the plot control, since all the Novelty (wilt susceptible) plants in a row wilted by early flowering stage. Seeds were sown mechanically in 1 m single rows, 30 cm apart, with 0.6 g of seeds from each RIL/cultivar per plot.

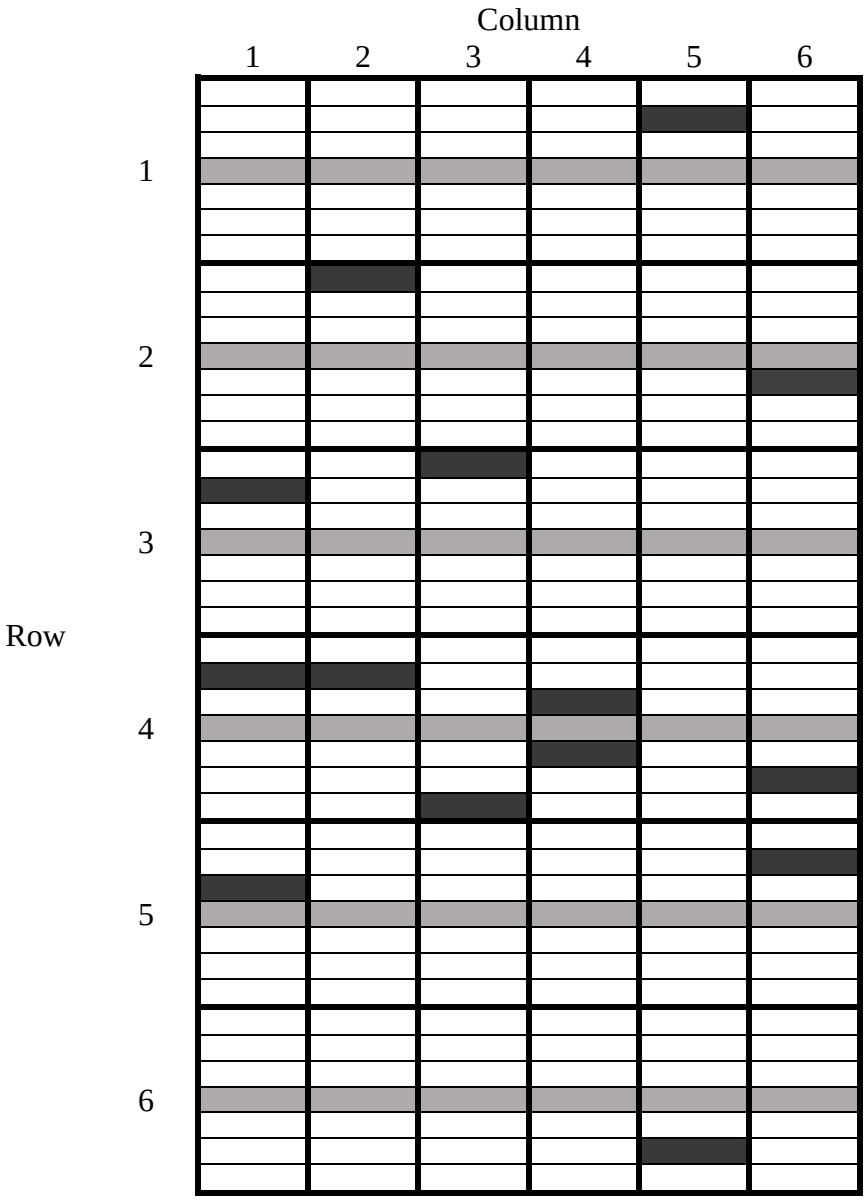


Figure 4.1 Field plot layout at the Saskatoon wilt nursery, 2013, with each whole plot split into seven subplots with a plot control in the center subplot (grey). Control subplots (black), were randomly allocated in the selected whole plots. Bison and Novelty were used as plot and subplot controls. The rest of the subplots (white) represented the RILs, Aurore and Oliver.

4.3.2.3 Disease Grading

Three disease assessments were conducted at each location each year, the first at the seedling stage (emergence of first pair of true leaves), the second at the early flowering stage and the third at the late flowering/green boll stage (Agriculture and Agri-Food Canada, 2011)) using the grading scale used in the preliminary disease screening (Chapter 3). The vigour of the RILs was recorded at the same time on a 1 – 5 scale: 1 the most

vigorous and 5 the least. The number of seedlings (Saskatoon wilt nursery only) and plant stand (rated 1-5) was recorded at the seedling stage with a full stand (high emergence and seedling survival) rated 1 and a very poor stand rated 5. Using the disease severity data from all 3 ratings, the AUDPC was calculated. Plants were not allowed to mature and were removed from the wilt nursery soon after the third assessment.

4.3.3 Data Analysis

Data collected from the growth chamber experiment, were analyzed using SAS 9.3 statistical software (SAS Institute Inc., Cary, NC, USA). All three isolates used in the RIL phenotyping under controlled conditions, deviated from a normal distribution (Shapiro – Wilk test) with respect to disease assessment at any stage, or for AUDPC, though normality was achieved with residuals for some variables. Similarly, some variables showed a significant difference in homogeneity of variance (Levene's test). Therefore, disease screening data of all three isolates were analyzed by fitting the data into a generalized linear mixed model (Proc Glimmix) with a Poisson distribution. Analysis of variance was conducted for the modelled data, combined for the three isolates as well as separately for each isolate. Pearson's correlation coefficients among disease grading at 21 days, AUDPC and height were assessed at the 5% significant level for all three isolates.

Phenotypic data from the wilt nurseries were adjusted with the use of the MAD analysis pipeline (You *et al.*, 2013), which used Activeperl 5.16 and SAS Version 9.3 software in alternating steps. However, the data from the Morden wilt nursery in 2014 was different from the other three nurseries and had low correlation. Therefore, it was removed from further analysis and the remaining three site years were used in the analyses. The data was analyzed in a mixed model for analysis of variance with regards to disease severity at the late flowering stage and AUDPC.

The data for the 160 RIL subset that were used in growth chamber phenotyping were extracted from the wilt nursery screening data, and the Pearson's correlation coefficient was determined for the controlled environment with the three isolates and the wilt nursery data. Finally, Pearson's correlation coefficients were calculated, using the wilt screening data obtained at early flowering stage in two wilt nurseries in northern France, Angiens and St. Aubin, and the disease severity data at early flowering stage in Saskatoon and Morden wilt nurseries.

Since significant differences were observed for the variables tested among the site years, principle component analysis was conducted for better representation of the data with respect to the location and year of assessment. Wilt, vigour and AUDPC were all considered in determining PCA.

4.4 Results

4.4.1 Selection of Three Isolates for RIL Phenotyping

Preliminary disease assessment data were used to select three isolates to screen the RIL population. Differential disease development on Aurore, Oliver, Bison and Novelty was used for the first selection of isolates. Since all isolates showed significant differences for the resistant and susceptible cultivars, moderately aggressive isolates were considered to screen the RIL population, to enable a more distinct variation in disease severity among the resistant and susceptible isolates.

Disease progression or disease reaction from 7 to 28 dai was recorded weekly to monitor disease development (Figure 4.2). Since a large spore count was required for inoculation of 160 RILs, spore production of each isolate in Czapek-Dox medium was also considered when selecting an isolate for RIL phenotyping.

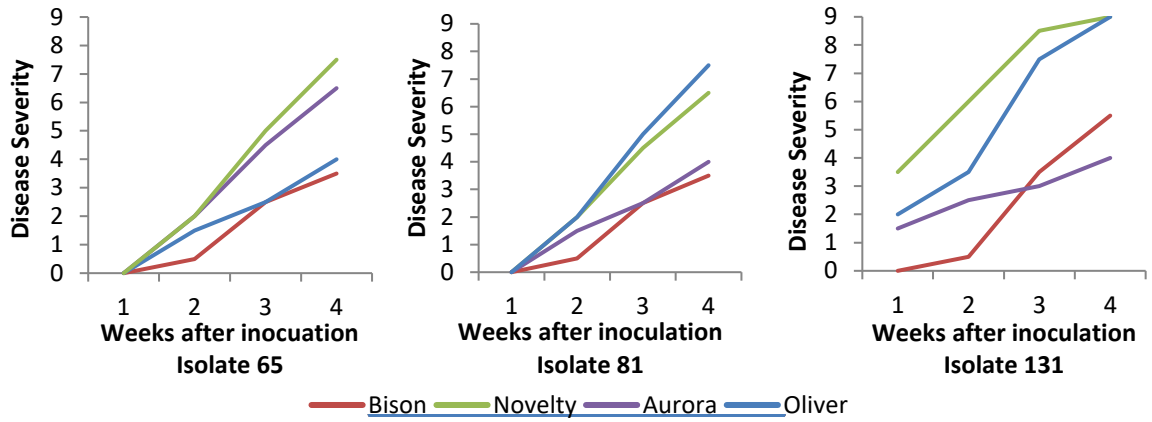


Figure 4.2 Disease severity of Bison (resistant), Novelty (susceptible), Aurore (resistant) and Oliver (susceptible) over the 4-week period with three isolates of *Fusarium oxysporum* f.sp. *lini*

Of the 17 isolates, 14 were collected from Treherne, MB, which posed the possibility that all might belong to the same physiological race, since a race structure study has not been conducted for the Canadian Prairies. However, only Isolate 65 of the remaining three isolates displayed a moderate level of aggressiveness, while both Isolates 66 and 159 showed weak reactions on the checks (Bison and Novelty) and parents of the RILs (Aurore and Oliver). Therefore, Isolate 65 was selected since it originated from a field at Indian Head, SK (Mpofu and Rashid, 2001). Based on these criteria, Isolates 65, 81 and 131 were selected for RIL phenotyping in the controlled environment.

4.4.2 RIL Phenotyping in the Growth Chamber

All three isolates and the RILs differed from each other with respect to disease severity at 21 and 28 days as well as AUDPC, based on RIL phenotyping data under controlled environment conditions. However, none of the isolate x RIL interactions were significant (Table 4.1).

Table 4.1 Analysis of variance and coefficient of variation (CV) for disease severity at 21 and 28 days after inoculation and area under the disease progress curve (AUDPC) of the 160 recombinant inbred lines with *Fusarium oxysporum* f.sp. *lini* Isolates 65, 81 and 131.

Variable	Effect	F value
Disease severity at 21 days	Isolate	5.26**
	RIL	2.46***
	RIL*Isolate	0.83
	CV	38.5
Disease severity at 28 days	Isolate	4.2*
	RIL	3.37***
	RIL*Isolate	0.94
	CV	25.33
AUDPC to 21 days	Isolate	15.28***
	RIL	2.21***
	RIL*Isolate	0.86
	CV	40.38
AUDPC to 28 days	Isolate	5.74**
	RIL	2.77***
	RIL*Isolate	0.89
	CV	35.77

* P < 0.05; ** P < 0.01; *** P <0.001

4.4.2.1 Isolate 65

The AUDPC and disease severity data deviated from a normal distribution, though residuals were normally distributed for AUDPC to 21 and 28 days, but not for disease severity. However, homogenous variance was not attained for any variable. Therefore, the data were fit to a generalized linear mixed model with a Poisson distribution, and all four variables (AUDPC and disease severity at 21 and 28 dai) were observed to be significant at p=0.05 (Table 4.2). The AUDPC and disease severity both showed a gradual increase over the RILs, rather than separation into clearly resistant and susceptible categories (Figure 4.3).

Table 4.2 *F* values of Type III tests of fixed effects of the analysis of variance from Proc GLIMMIX for disease severity and area under the disease progress curve (AUDPC) at 21 and 28 days after inoculation for the 160 F_8 lines of Aurore x Oliver recombinant inbred line population and coefficients of variation (CV) with Isolate 65.

Variable	F Value	CV
Disease severity at 21 days	1.77***	46.19
Disease severity at 28 days	6.69***	17.96
AUDPC at 21 days	1.43***	51.02
AUDPC at 28 days	2.08***	40.94

*** P <0.001

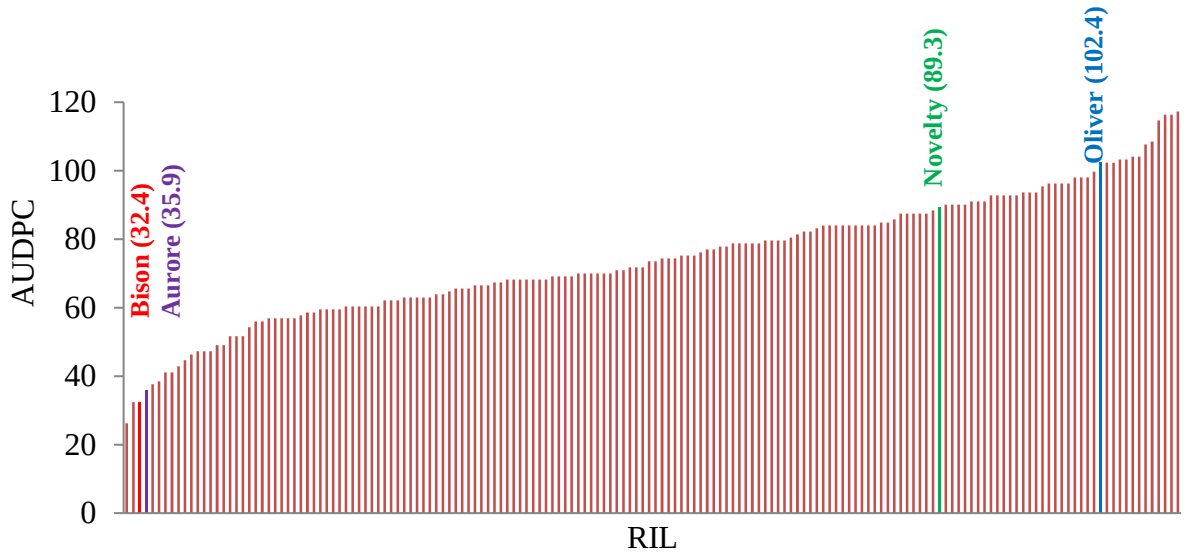


Figure 4.3 The AUDPC of the 160 F_8 lines of the Aurore x Oliver recombinant inbred line population, at 21 days after inoculation, with Isolate 65 under controlled environment, along with resistant and susceptible checks (Bison and Novelty) and parents (Aurore and Oliver).

The RILs were severely diseased (rating of >6) and the AUDPC high at 28 days. Most of the RILs were rated 7 at 28 dai, indicating the high level of susceptibility of the RILs to Isolate 65 with fewer than 10 lines that had disease rating of ≤ 3. Therefore, to observe greater variation among the RILs, disease severity and AUDPC at 21 days were considered for analysis (Figure 4.4).

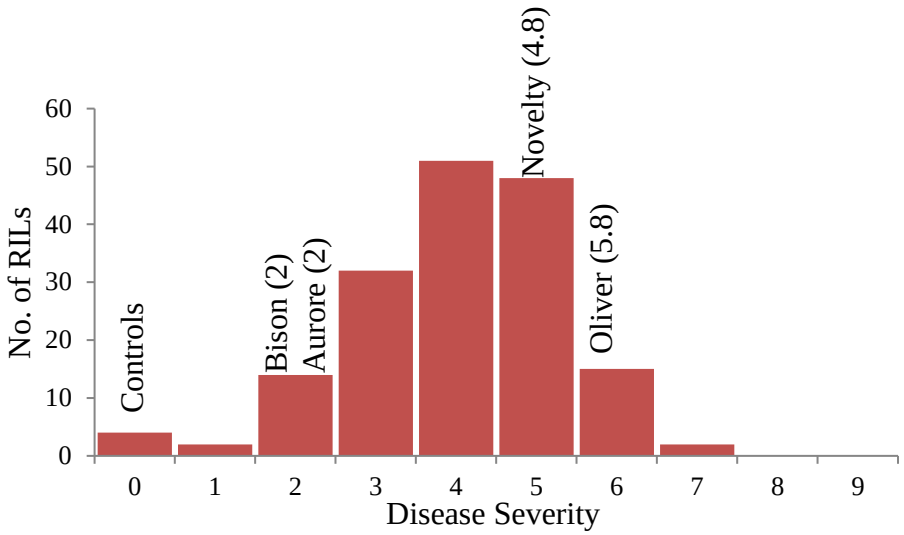


Figure 4.4 Frequency distribution of disease severity of the Aurore X Oliver recombinant inbred line population at 21 days after inoculation with Isolate 65 in a controlled environment, along with resistant (Aurore) and susceptible (Oliver) checks. Control pots were not inoculated.

Correlations between disease severity, AUDPC and height at 21 dai were calculated. Disease severity and AUDPC were highly correlated. An inverse, moderate correlation was detected between both disease severity and plant height at 21 dai and AUDPC and plant height at 21 dai. All correlations were significant (Table 4.3).

Table 4.3 Pearson's correlation coefficients for disease severity, area under the disease progress curve (AUDPC) and height at 21 days after inoculation with Isolate 65 under a controlled environment.

	AUDPC	Height
Disease severity	0.91***	-0.47***
AUDPC		-0.42***

*** $P < 0.001$

4.4.2.2. Isolate 81

Similar to Isolate 65, a normal distribution was achieved with residuals for AUDPC at 28 days and disease severity at 21 days at the 5% significance level, and the variance for all variables, except disease severity at 28 days, deviated from homogeneity. Therefore, the data were fit to a generalized linear mixed model and tested for variance of the RILs and to observe variation with respect to AUDPC and disease severity at 21 and 28 dai (Table 4.4).

Table 4.4 *F* values of Type III tests of fixed effects of analysis of variance from Proc GLIMMIX for disease severity, area under the disease progress curve (AUDPC) and coefficients of variation (CV) at 21 and 28 days after inoculation for the 160 F_8 of the Aurore x Oliver recombinant inbred line population with Isolate 81.

Variable	F Value	CV
Disease severity at 21 days	2.91***	31.60
Disease severity at 28 days	4.46***	19.55
AUDPC to 21 days	1.78***	49.86
AUDPC to 28 days	2.71***	31.76

*** $P < 0.001$

Similar to the results of Isolate 65, the RILs showed gradual variation from resistant to susceptible for both AUDPC (Figure 4.5) and disease severity. Distribution of the disease rating at 28 dai was skewed to the right, with the majority of the lines rated 6 and 7 at 28 dai (data not shown). While the AUDPC of individual RILs was slightly lower for isolate 81 than Isolate 65, there were also fewer resistant lines and more moderately resistant lines (rated 4-6) than with Isolate 65 at 28 dai. Thus, to acquire a near normal distribution, disease severity and AUDPC at 21 days was considered (Figure 4.6).

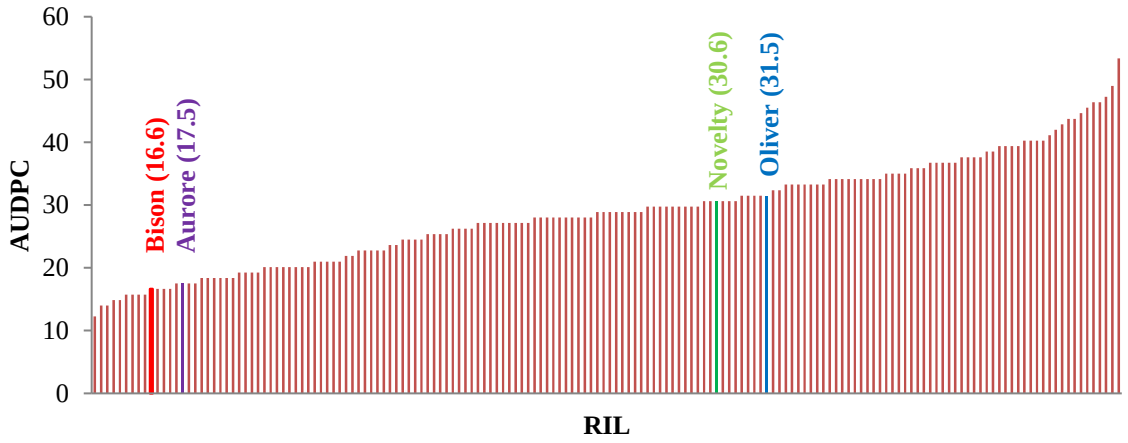


Figure 4.5 The AUDPC of the 160 F_8 lines of the Aurore x Oliver recombinant inbred line population, at 21 days after inoculation, with Isolate 81 under a controlled environment including parents (Aurore and Oliver) and the resistant and susceptible checks (Bison and Novelty).

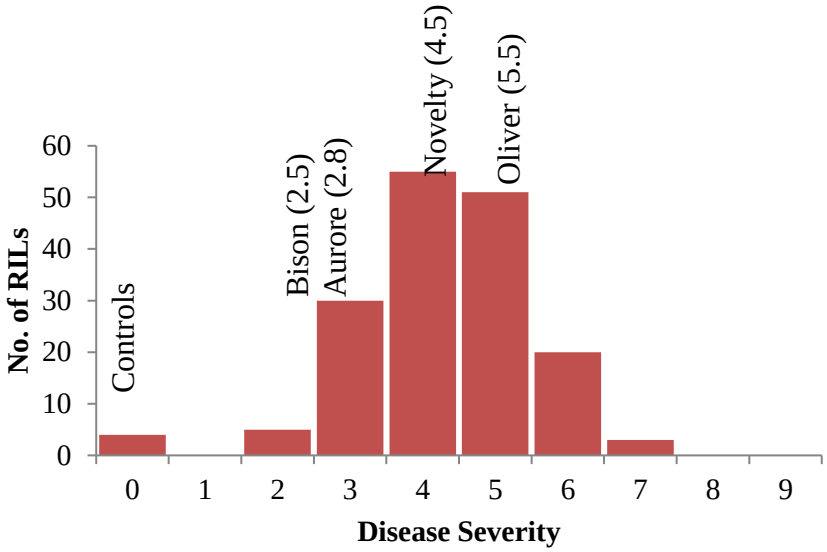


Figure 4.6 Frequency distribution of disease severity of the Aurore X Oliver recombinant inbred line population at 21 days after inoculation with Isolate 81 in a controlled environment, along with resistant (Aurore) and susceptible (Oliver) checks. Control pots were not inoculated.

Sixty-five percent of the RILs used for phenotyping with Isolate 81 showed disease severity of between four and six; only three lines had a rating of seven or high at 21 dai compared to 40% of the RILs that had an average disease rating of 7 or higher at 28 dai.

Pearson's correlation coefficients were determined for Isolate 81 to identify the relationship between AUDPC, disease assessment and height at 21 dai. Similar to Isolate 65, height at 21 days was negatively correlated with AUDPC and disease rating at 21 days, but at a lower level (Table 4.5).

Table 4.5 Pearson's correlation coefficients for disease severity, area under the disease progress curve (AUDPC) and height at 21 days after inoculation with Isolate 81 in a controlled environment.

	AUDPC	Height
Disease severity	0.84***	-0.33***
AUDPC		-0.27***

*** $P < 0.001$

4.4.2.3. Isolate 131

The AUDPC for RILs inoculated with Isolate 131 was not normally distributed. The same as with the other two isolates, when residuals were tested the data was normally

distributed (Shapiro – Wilk test) for disease severity at 21 days and AUDPC at 21 and 28 days but not for the other variables. The AUDPC at 21 and 28 days had homogenous variance, while disease severity at 21 and 28 days had heterogeneous variance, at the 5% significant level as indicated by Levene's test of homogeneity. Therefore, the data was fit to a generalized linear mixed (GLIMMIX) model, in which RILs were significant, for both AUDPC and disease grading at 21 and 28 days, at the 5% probability level (Table 4.6). The AUDPC and disease severity at 21 days had similar trends as with the other two isolates (Figure 4.7).

Table 4.6 *F* values of Type III tests of fixed effects of analysis of variance from Proc GLIMMIX for disease severity, area under the disease progress curve (AUDPC) and coefficients of variation (CV) at 21 and 28 days after inoculation for the 160 F_8 lines of the Aurore x Oliver recombinant inbred line population, with Isolate 131 under controlled conditions.

Variable	F Value	CV
Disease severity at 21 days	1.21^{ns}	45.31
Disease severity at 28 days	1.25*	32.16
AUDPC to 21 days	1.36**	49.98
AUDPC to 28 days	1.37**	39.33

ns – not significant; * $P < 0.05$; ** $P < 0.01$

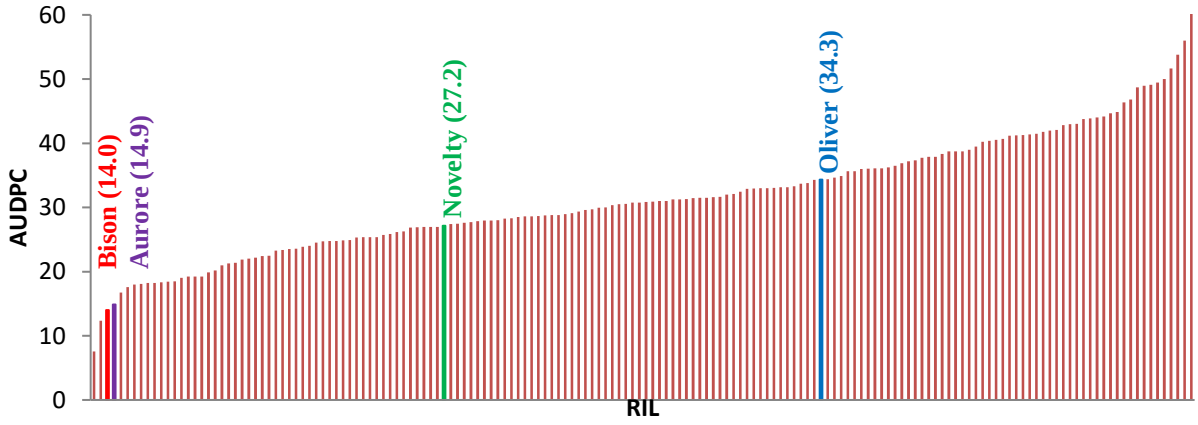


Figure 4.7 Area under the disease progress curve for the 160 F_8 recombinant inbred lines derived from the cross of Aurore x Oliver, at 21 days after inoculation, with Isolate 131 under controlled conditions. Parents Aurore and Oliver and the resistant (Bison) and susceptible (Novelty) checks included.

Pearson's correlation coefficients were calculated to identify the relationship between AUDPC, disease severity at 21 dai and plant height. Height was poorly correlated with AUDPC, and there was no correlation between height and disease severity at 21 dai (Table 4.7).

Table 4.7 Pearson's correlation coefficients between disease severity, area under the disease progress curve (AUDPC) and height at 21 days after inoculation with Isolate 131 under controlled conditions.

	AUDPC	Height
Disease severity	0.86***	-0.06^{ns}
AUDPC		-0.21***

ns – not significant; *** $P < 0.001$

Of the three isolates, Isolate 131 developed the highest AUDPC, with the greatest number of RILs developing severe disease (>8 28 dai). However, at 21 dai, over one hundred RILs had an average disease rating of 5 or 6 (Figure 4.8).

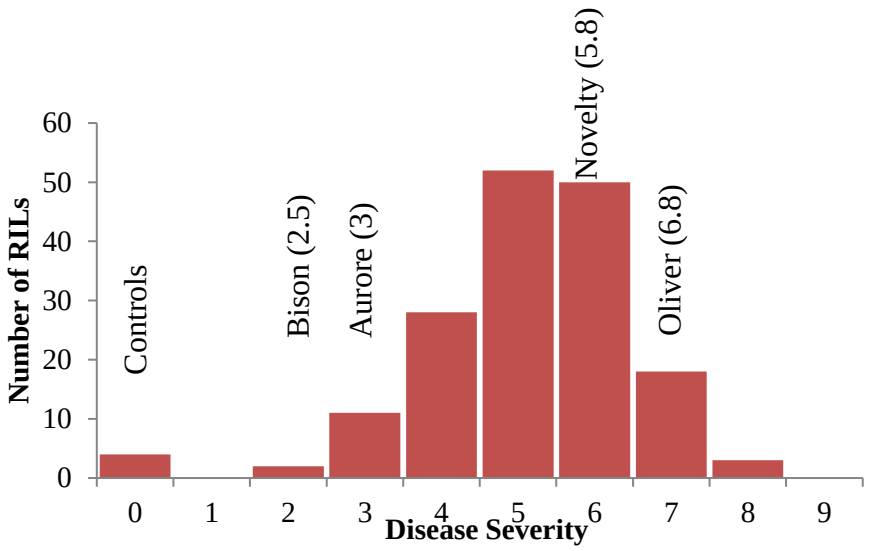


Figure 4.8 Frequency distribution of disease severity of 160 F_8 lines of the Aurore x Oliver recombinant inbred line population at 21 days after inoculation with Isolate 131. Mean scores of Bison, Novelty, Aurore and Oliver indicated. Control pots were not inoculated.

4.4.3 RIL Phenotyping in Wilt Nursery

The MAD pipeline (You *et al.*, 2013) was used to adjust the data from the wilt nursery experiment based on plot and subplot controls. The three site years were considered different environments. Environment by genotype interaction was significant for both AUDPC and disease severity at the late flowering/green boll stage, as well as all other variables (Table 4.8). Coefficients of variation were low for AUDPC and disease severity, indicating the variation among the three site years was comparatively low. The sum of squares was highest for genotype (RIL) and lowest for site year.

Table 4.8 Sum of squares (SS) and *F* values from disease severity and analysis of variance of area under the disease progress curve (AUDPC) at late flowering stage, of site year, genotype and their interactions and their corresponding coefficients of variation (CV).

Measurement	Source	SS	F Value
AUDPC	Site year	169602.84	212.77***
	Genotype	4150292.89	51.30***
	Site year*Genotype	769590.83	4.76***
	CV		9.28
Disease severity	Site year	122.03	133.16***
	Genotype	2876.18	30.92***
	Site year*Genotype	844.76	4.54***
	CV		11.78

*** $P < 0.001$

There was a gradual increase in AUDPC and disease severity of the RILs at both locations, similar to that observed in the controlled environment. Since there was a significant difference among site years for disease severity, they were not combined. All three site years showed a similar pattern for both AUDPC and disease severity [Figure 4.9 (a - f)].

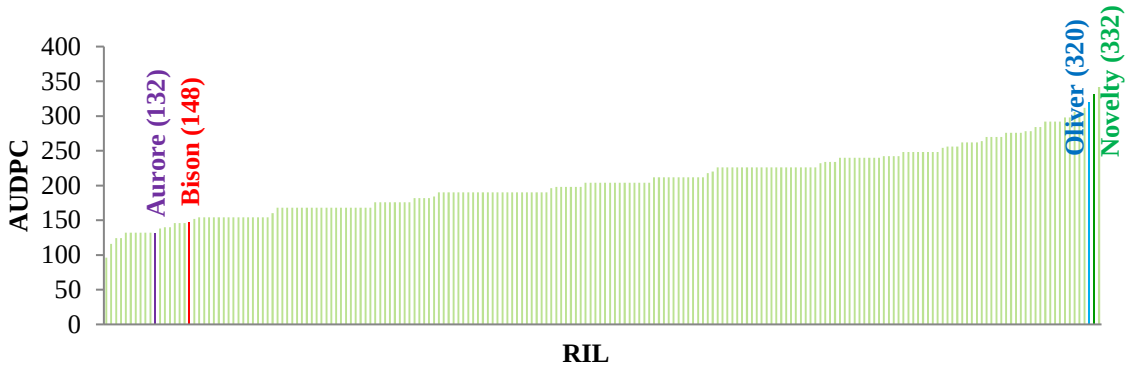


Figure 4.9 (a) Area under the disease progress curve (AUDPC) of 200 F_8 lines of the Aurore x Oliver recombinant inbred population, parents (Aurore (R) and Oliver (S)) and checks (Bison (R) and Novelty (S)) at the Saskatoon wilt nursery in 2013.

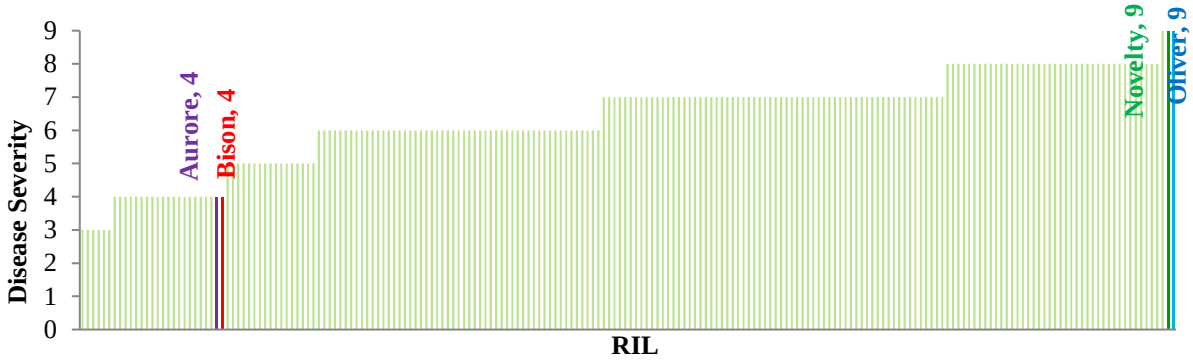


Figure 4.9 (b) Disease severity of 200 lines from the F_8 generation of the Aurore x Oliver recombinant inbred line population, parents (Aurore (R) and Oliver (S)) and checks (Bison (R) and Novelty (S)) at the Saskatoon wilt nursery in 2013.

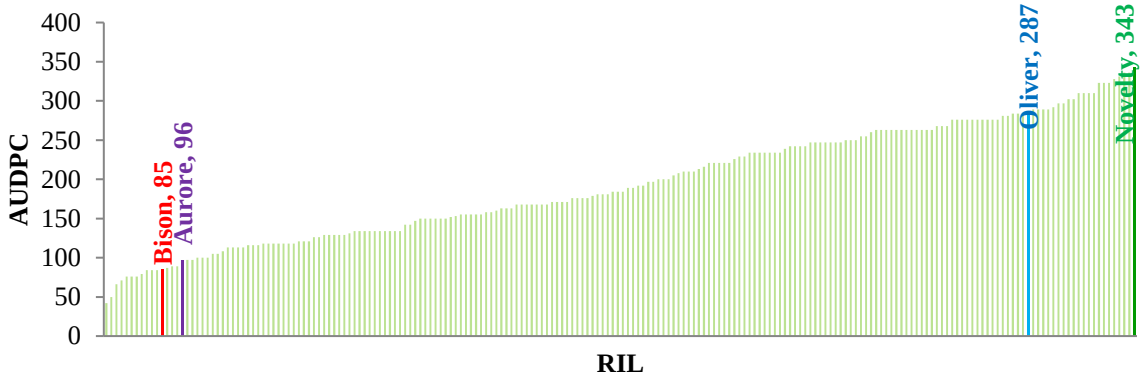


Figure 4.9 (c) Area under the disease progress curve (AUDPC) of 200 F8 lines of the Aurore x Oliver recombinant inbred population, parents (Aurore (R) and Oliver (S)) and checks (Bison (R) and Novelty (S)) at the Saskatoon wilt nursery in 2014.

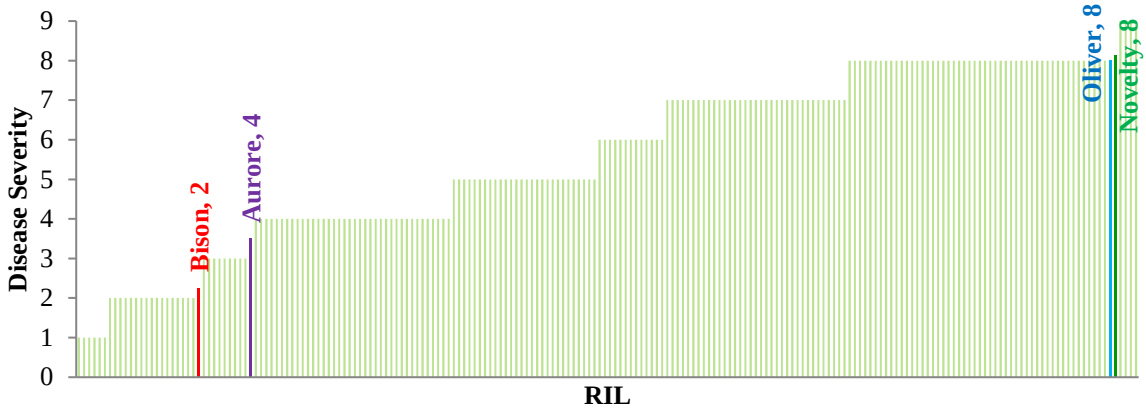


Figure 4.9 (d) Disease severity of 200 lines from the F8 generation of the Aurore x Oliver recombinant inbred line population, parents (Aurore (R) and Oliver (S)) and checks (Bison (R) and Novelty (S)) at the Saskatoon wilt nursery in 2014.

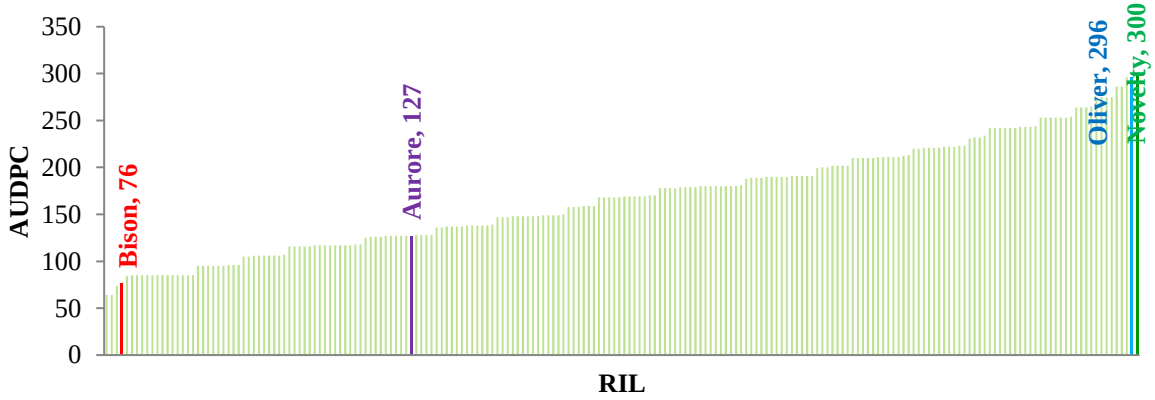


Figure 4.9 (e) Area under the disease progress curve (AUDPC) of 200 F8 lines of the Aurore x Oliver recombinant inbred population, parents (Aurore (R) and Oliver (S)) and checks (Bison (R) and Novelty (S)) at the Morden wilt nursery in 2013.

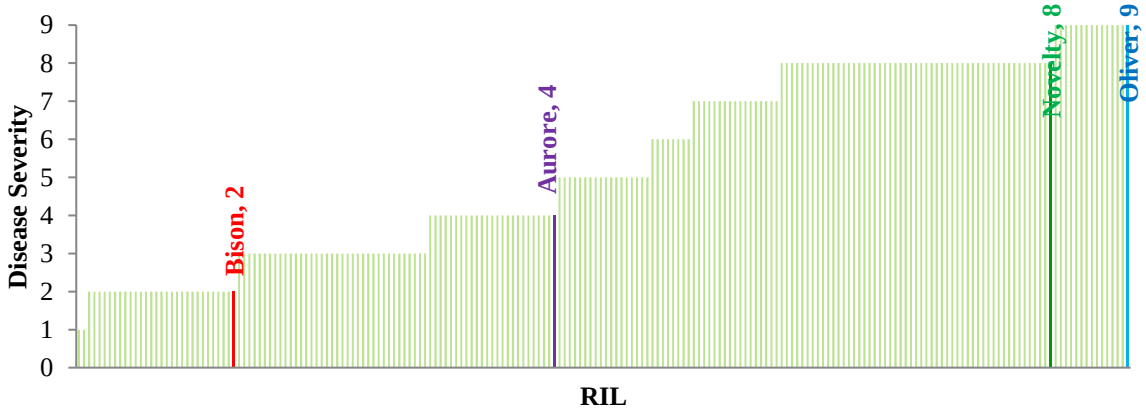


Figure 4.9 (f) Disease severity of 200 lines from the F8 generation of the Aurore x Oliver recombinant inbred line population, parents (Aurore (R) and Oliver (S)) and checks (Bison (R) and Novelty (S)) at the Morden wilt nursery in 2013.

Pearson's correlation coefficients were also calculated for the disease severity ratings at four site years in France: Angiens wilt nurseries in 2011-2013 (3 years) and St. Aubin, 2012, for all 200 RILs (Table 4.9).

Table 4.9 Pearson's correlation coefficients for disease severity of RILs of the cross Aurore x Oliver at the early flowering stage in the Saskatoon wilt nursery in 2013 and 2014, Morden wilt nursery in 2013 and the French wilt nurseries in Angiens from 2011 to 2013 and St. Aubin in 2012.

	Angiens 2011	Angiens 2012	Angiens 2013	StAubin 2012	Morden 2013	Saskatoon 2013
Angiens 2012	0.82***					
Angiens 2013	0.82***	0.83***				
St. Aubin 2012	0.80***	0.87***	0.80***			
Morden 2013	0.60***	0.49***	0.53***	0.52***		
Saskatoon 2013	0.53***	0.50***	0.45***	0.52***	0.52***	
Saskatoon 2014	0.56***	0.56***	0.50***	0.55***	0.55***	0.53***

*** $P < 0.001$

Moderate but significant correlations were observed between French and Canadian wilt nursery ratings, showing similar resistance patterns among the RILs tested in the two countries. Rating of the French wilt nurseries were conducted at the early to mid-flowering stage. Therefore, the data from the second rating conducted at early flowering stage was considered for Canadian wilt nurseries in determining Pearson's correlation coefficient between the wilt nurseries in the two countries.

Two principle components that explained 82% of the variation were identified for the wilt nursery screening of the RILs. Data separated into two distinct groups based on location, while data from the two years showed no distinct separation (Figure 4.10).

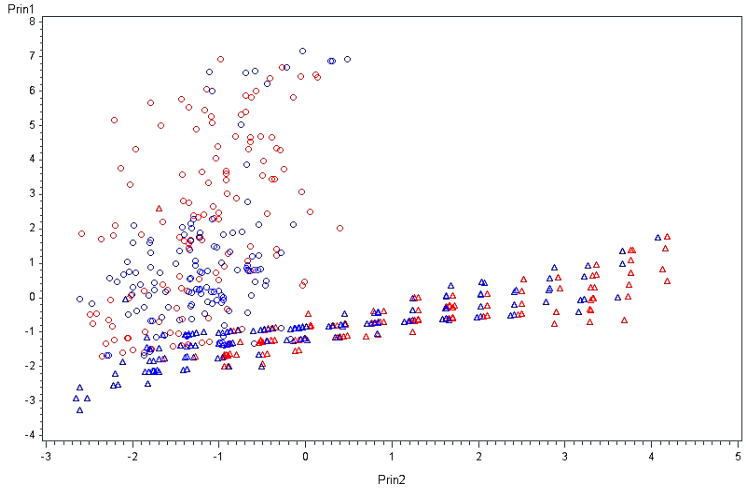


Figure 4.10 Principle component analysis of disease severity of the RILs of the cross Aurore x Oliver from wilt nurseries at Saskatoon (Δ) and Morden wilt nursery (◯) in 2013 (▬) and 2014 (▬).

Pearson's correlation coefficients were determined for the subset of 160 RILs between AUDPC and disease severity at the final rating (late flowering/green boll stage in the wilt nursery and 21 dai under controlled conditions). All correlations were moderate but significant. Correlations were higher between the two nurseries in 2013 and Isolates 65 and 81 (Table 4.10).

Table 4.10 Pearson's correlation coefficients for area under the disease progress curve (AUDPC) in the Saskatoon and Morden wilt nurseries in 2013 and 2014 at late flowering/green boll stage and with Isolates 65, 81 and 131, 21 days after inoculation under controlled conditions for 160 F_8 RILs of the cross Aurore x Oliver.

	Saskatoon 2013	Morden 2013	Saskatoon 2014	Isolate 65	Isolate 81
Morden 2013	0.62***				
Saskatoon 2014	0.56***	0.49***			
Isolate 65	0.39***	0.27***	0.51***		
Isolate 81	0.36***	0.19*	0.34***	0.48***	
Isolate 131	0.38***	0.31***	0.35***	0.28***	0.36***

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

4.5 Discussion

Isolate selection for RIL phenotyping was based on four criteria to select the most resistant lines under controlled environmental conditions and to obtain the most varied disease response. Disease response of Aurore and Oliver with the selected isolates differed, indicating the RIL population generated was segregating for *Fol* resistance to the three isolates.

The three isolates selected for RIL phenotyping were moderately aggressive, meaning a gradual increase in disease symptoms was observed over the duration of the study under controlled conditions. This resulted in a consistent separation of RILs based on resistance level. Czapek – Dox medium is a minimal medium and abundant sporulation of other *formae speciales* of *Fusarium oxysporum* have been reported (Chittem and Kulkarni, 2008; Islam, 2015). The *Fol* isolates also sporulated well on Czapek-Dox media, which ensured sufficient amounts of spores for inoculation.

Races of *Fol* have not been identified on the Canadian prairies, but minor gene differences that cause variability in aggressiveness exist among isolates, which are more distinctly observable with isolates from different locations (Kroes et al., 1997, Saharan *et al.*, 2005). Of the 17 isolates in the preliminary study, only three were collected from locations other than Treherne, MB: Isolates 65, 66 and 159 (Mpofu and Rashid, 2001). Hence, Isolate 65 which fit with the other criteria, was selected along with Isolates 81 and 131. Isolate 65, collected from Indian Head was selected to increase the chances that the isolates represent different pathotypes. Thus, the three isolates were expected to result in variable disease reactions on the RILs based on that observed on the parents.

The three isolates and the individual RILs differed in disease severity and AUDPC at 21 and 28 days. However, there was no significant isolate x RIL interaction for any of the four variables tested and the coefficients of variation were moderate (CV = 25.3 – 40.4%).

Contrary to this, Kroes *et al*. (1997) identified *Fol* races, using 16 cultivars and 25 isolates, which resulted in significant isolate x cultivar interactions. However, most of the variation was attributed to isolate and cultivar differences and the interaction was considered due to minor gene effects. In the present study, only three isolates were used with two replicates and one repeat. Inclusion of more isolates and replicates could increase the precision of the data.

With each isolate used under the controlled conditions, the variation in disease severity and AUDPC of the RILs was similar. The RILs were different with respect to AUDPC and disease severity at 21 and 28 days for all three isolates, except for disease severity at 21 days with Isolate 131. In a segregating population derived from genetically different parents it is expected that the progeny will vary for resistance. High coefficients of variation were observed with disease severity at 21 days compared to 28 days. However, at 28 dai, severe wilting (mean rating $\geq$7) was observed for 87, 64 and 98 of the lines inoculated with Isolates 65, 81 and 131, respectively, and less than ten lines resistant (mean rating $\leq$3). Therefore, to obtain variation in disease response, the data from 21 dai were considered. Inclusion of more replicates and the susceptible check in each pot might reduce the variation observed among replicates.

The height of the affected plants was influenced by flax wilt, thereby resulting in an inverse correlation between height and disease severity, indicating the adverse effect of *Fol* on plant growth. Final disease severity and the AUDPC, was considered an indication of the level of resistance of the RILs. Many lines were severely diseased by all three isolates (rated $\geq$ 8), although Isolate 131 caused the most severe disease and Isolate 81 the least.

Variation in disease severity may be explained by the climate data from the three site years (Table 4.11). Maximum and mean temperatures have been reported to be positively correlated to wilt incidence in chickpea (Mina and Dubey, 2010). Similarly, temperatures

in the range of 24 to 28°C increase wilt incidence and severity in flax as well as in other crops (Saharan and Mehta, 2005; Muskett and Colhoun, 1947). While the temperature data from the three site years were similar to the thirty-year average, total precipitation at Saskatoon in 2013 and 2014 was lower than the long-term average. Dry soil conditions have been reported to promote flax wilt (Kommedahl *et al.*, 1970). Thus, low soil moisture levels may have resulted in the severe disease observed at Saskatoon.

Table 4.11 Monthly mean and maximum temperatures (°C) and total precipitation (mm) in Saskatoon, SK and Morden MB in 2013 and 2014 for the months the plants were in wilt nursery (Government of Canada/Climate.) and the 30 year average at the two locations.

		Morden			**Saskatoon**		
Year	**Month**	Average of Mean Temp (°C)	Average of Max Temp (°C)	Total Precipitation (mm)	Average of Mean Temp (°C)	Average of Max Temp (°C)	Total Precipitation (mm)
2013	May	10.8	16.6	170.5	-	-	-
	June	18.2	24.1	23.6	-	-	-
	July	19.5	24.9	86.9	17.4	23.6	35.2
	August	19.5	25.9	33.7	18.9	26.4	14.7
	September	-	-	-	22.9	15.2	14.9
2014	May				-	-	-
	June				-	-	-
	July				18.3	24.5	44.5
	August				17.9	24.6	18.5
	September				12.4	19.4	10.7
30-year average (1981-2010)	May	12.7	19.3	58.4	-	-	-
	June	17.6	23.4	92.9	-	-	-
	July	20	25.6	79.4	19.0	25.7	53.8
	August	19.5	25.6	70.8	18.2	25.2	44.4
	September	-	-	-	12.0	18.4	38.1

In a few instances, disease severity was observed to be lower than the previous disease rating, suggesting adult plant disease resistance. However, with more thorough observation it was apparent that the plants with high disease severity in the previous rating had died and only the more resilient plants survived. The rows were thinning but were scored lower for wilt and plants were more vigorous.

Site year and genotype (RIL) as well as their interaction were observed to be significant for disease severity and AUDPC at the late flowering stage. Significant genotype by

environment interaction may suggest inconsistent expression of genotype in different environments due to environmental influences (Olaniyi *et al*., 2013). Thus, variable disease severity scores can be expected for the same RIL in different environments. However, most of the variation can be attributed to the genotype based on sum of squares, which explained 75% and 82% of the variation observed with respect to AUDPC and disease severity, while site year (environment) explained only 4% and 3% of the variation, respectively. A similar pattern was observed by Kroes (1997) where significant cultivar by environment effects were observed but the magnitude was lower than the cultivar or environment effects. Kroes (1997) also reported higher genetic effect (cultivar) than environment effect, the later the stage the assessment was conducted.

Significant correlations among locations in Canada and France, as well as the controlled environment studies indicated the RILs developed the disease in a similar manner across environments. The higher correlation between Morden and Saskatoon wilt nurserries in 2013, compared to the Saskatoon wilt nursery in 2013 and 2014 may be an indication that the two locations have a similar pathogen structure. In the experiment by Kroes *et al.* (1997) it was reported that greater difference was observed when the cultivars were inoculated with the isolates from the different continents than from the same location. This suggests that minor gene differences occur among isolates from different continents. However, they also stated that the aggressiveness of the Canadian isolates (collected from Morden) were comparable to European isolates, supporting the moderate correlation observed between French and Canadian wilt nurseries in this study.

Chapter 5: Inheritance of Fusarium Wilt Resistance in Flax

5.1 Abstract

Wilt disease reaction caused by *F. oxysporum* f. sp. *lini* (*Fol*), on recombinant inbred lines (RILs) at the F_8 generation, from a cross between flax cultivars, Aurore (A) and Oliver (O), was used to study the inheritance of resistance. Segregation of resistance was determined for AO RILs grown in wilt nurseries and under controlled conditions screened with three *Fol* isolates. A 3:1 ratio of susceptible to resistant RILs was observed with Isolates 81 and 131 indicating the inheritance of resistance to flax wilt was conditioned by two independent recessive genes. The RILs were divided into two groups, which also complied with the 3:1, susceptible: resistant ratio, in a dendrogram using the disease severity data at 14, 21 and 28 days after inoculation (dai) in a controlled environment and at seedling, early flowering and late flowering flax growth stages at the Saskatoon wilt nursery in 2013 and 2014 and at the Morden wilt nursery in 2013. The heritability of resistance to wilt, calculated on disease severity at the late flowering stage, was observed to be high (0.90). High heritability indicated a high level of genotypic variation contributing to phenotypic variation. Understanding the inheritance patterns and locating the genes/QTLs contributing to resistance to flax wilt facilitates more efficient selection of wilt resistant phenotypes in the breeding of flax.

5.2 Introduction

Wilt caused by *Fusarium oxysporum* f. sp. *lini* (Bolley) Snyder & Hanssen (*Fol*), is a major disease of flax (*Linum usitatissimum* L.) and yield loss due to the disease was estimated to be between 30 and 50%, with 100% yield loss for susceptible cultivars (Kommedahl, 1970). Seed treatments, crop rotation, fungicide application and soil treatment can be used to reduce the pathogen population, although due to the soil-borne nature of the pathogen and its ability to survive in soil for prolong periods, it is difficult to eliminate the pathogen or achieve 100% disease control. The most efficient and environmentally friendly method of disease control is achieved by developing wilt resistant cultivars.

Flax lines are regularly screened for resistance to flax wilt in breeding programs, and are required to have at least moderate resistance to be registered as a cultivar for production in Canada. However, screening a large number of flax lines and cultivars exhibiting resistance to wilt can be tedious, expensive and time consuming. Marker assisted selection (MAS) can be used as an alternative, to screen a large number of individuals for the presence of resistance genes, using molecular markers associated with these genes. This can increase the efficiency of selection in breeding and facilitate gene pyramiding to develop cultivars with broad-spectrum resistance (Joshi and Nayak, 2010).

The genetic basis of flax wilt resistance is yet to be established, with contradictory observations reported. A prominent hypothesis is that flax wilt resistance is polygenic (Kommendahl et al., 1970; Kroes, 1997). Supporting the assumption of polygenic resistance, is the existence of an unaccountable number of *Fol* races, as reported by Kroes et al. (1997). However, wilt resistance due to two dominant genes in an F_2 population of a cross between two Chinese flax cultivars has been reported by Wu et al., (2015), while Spielmeyer et al., (1998b) reported two individual genes with additive effects conferring

resistance in a doubled haploid population from a cross between two Australian cultivars. Major gene resistance has not been observed in Canadian cultvars.

Resistance against wilt in crops such as —cucumber, melon and chickpea, caused by various *formae speciales* of *F. oxysporum*, was reported to be monogenic or digenic (Zhang et al., 2014; Oumouloud et al., 2013; Perchepied et al., 2005; Gowda et al., 2009). However, the number of pathogen pathotypes/ isolates present in these studies was also limited, though they have suggested that race-specific resistance, along with partial resistance through multiple genes effective against some virulent races, is responsible for disease control. The objective of this study was to determine the inheritance pattern of flax wilt resistance.

5.3 Methodology

5.3.1 Statistical Analysis

The SAS 9.3 statistical software (SAS Institute Inc., Cary, NC, USA) was used in the data analysis of this study. Wilt screening data of Aurore (moderately resistant to flax wilt; spring, fibre type) x Oliver (susceptible to flax wilt; winter, linseed type) RIL population was used to perform chi-squared tests, to test the inheritance patterns of 1:1, 1:2:1, 1:15, 9:7 and 3:1 ratios for resistance to flax wilt (data from Chapter 4). A disease severity score of $\leq$4 was considered resistant and >4 was considered susceptible at 21 days for the 160 RILs under controlled conditions for this test. Similarly, disease severity of $\leq$3 were considered resistant for all three growth stages for wilt nursery data from Saskatoon and Morden to determine inheritance pattern. A higher disease severity rating was considered for controlled environment screening, since a conducive environment for *Fol* was provided in single isolate screening.

Using the same subset of 160 RILs, cluster analysis was performed to generate a dendrogram. Unweighted pair grouping by mathematical average algorithms (UPGMA)

was used here, with Ward's minimum-variance clustering method. Disease severity at 14, 21 and 28 days under controlled conditions and disease severity at seedling, early flowering and late flowering stages in the wilt nurseries were used to produce the dendogram.

Using the results from ANOVA analysis of wilt scores at late flowering stage from the wilt nurseries and 21 days after inoculation (dai) under control environment, broad sense heritability was calculated using the following formula (Conner and Hartl, 2004). Additive effects were not considered in the calculation. Each site year was considered a separate replication in this calculation since a modified augmented design (MAD) was used.

$$\textit{Genetic Variance } (V_g) = \frac{\text{Genotype mean square - error mean square}}{\text{Number of replications}}$$

$$\textit{Environmental Variance } (V_e) = \text{Error mean square}$$

$$\textit{Phenotypic Variance } (V_p) = V_g + V_e$$

$$\textit{Heritability } (H^2) = \frac{V_g}{V_p}$$

Single nucleotide polymorphism (SNP) data of the RIL population generated by next generation sequencing was provided and was intended to be used in a QTL analysis. However, 99.9% of the data showed segregation distortion, and could not be used in a reliable QTL analysis (Appendix III). Four simple sequence repeats (SSR) markers previously identified to be linked with flax wilt resistance was also used to genotype 92 RILs, although none of them were identified to be associated with flax wilt resistance of the AO population (Appendix IV).

5.4 Results

5.4.1 Inheritance of Resistance to Flax Wilt

Based on the phenotypic data it was observed that the population deviated from an expected 1:1 segregation ratio of monogenic inheritance in both environments. Phenotypic

data with the three isolates were tested separately and Isolates 81 and 131 both showed non-significant p-values indicating that they fit a 3:1 ratio of inheritance of two independent genes (Table 5.1). However, segregation for Isolate 65 did not conform to the 3:1 ratio. Wilt nursery data in year 2013 showed significant chi-square values when tested for segregation ratios of 1:1 and 3:1, although the flax wilt ratings at early flowering stage at Saskatoon wilt nursery in 2014 complied with the 3:1 ratio. The phenotypic data did not conform to the other genes ratios tested at p=0.05.

Table 5.1 Chi-squared test to fit a two gene model of the 3:1 flax wilt susceptible : wilt resistant expected in the RIL population developed from the flax wilt resistant cultivar Aurore and wilt susceptible cultivar Oliver under controlled conditions with Isolates 65, 81 and 131 in the Saskatoon wilt nursery in 2013 and 2014 and the Morden wilt nursery in 2013.

Isolate/ Site year	Observed		Expected		χ^2	P value
	Suceptible	Resistant	Susceptible	Resistant		
65	97	63	120	40	17.63	<0.0001
81	116	44	120	40	0.53	0.47
131	129	31	120	40	2.70	0.10
Saskatoon 2013	186	14	150	50	24.11	<0.0001
Morden 2013	134	66	150	50	6.83	0.009
Saskatoon 2014	139	61	150	50	3.23	0.072

Cluster analysis identified two major groups based on disease severity in wilt nurseries and under controlled conditions (Figure 5.1). Cluster 1 represented 35 RILs that displayed greater resistance to flax wilt than RILs in Cluster 2, in the wilt nurseries and under controlled conditions. Cluster 2 consisted of flax wilt susceptible RILs, while there were many smaller groups observed within these two major clusters. Similar clustering was observed with isolates 81 and 131 under controlled environment and at Saskatoon wilt nursery in 2014. Most RILs separated into resistant and susceptible groups were identical

among the site years and the single isolate screening. The separation of RILs into two clusters of the dendrogram was in accordance with a 3:1 ratio of wilt susceptible to resistant, with a chi-squared value of 0.833 ($p = 0.36$).

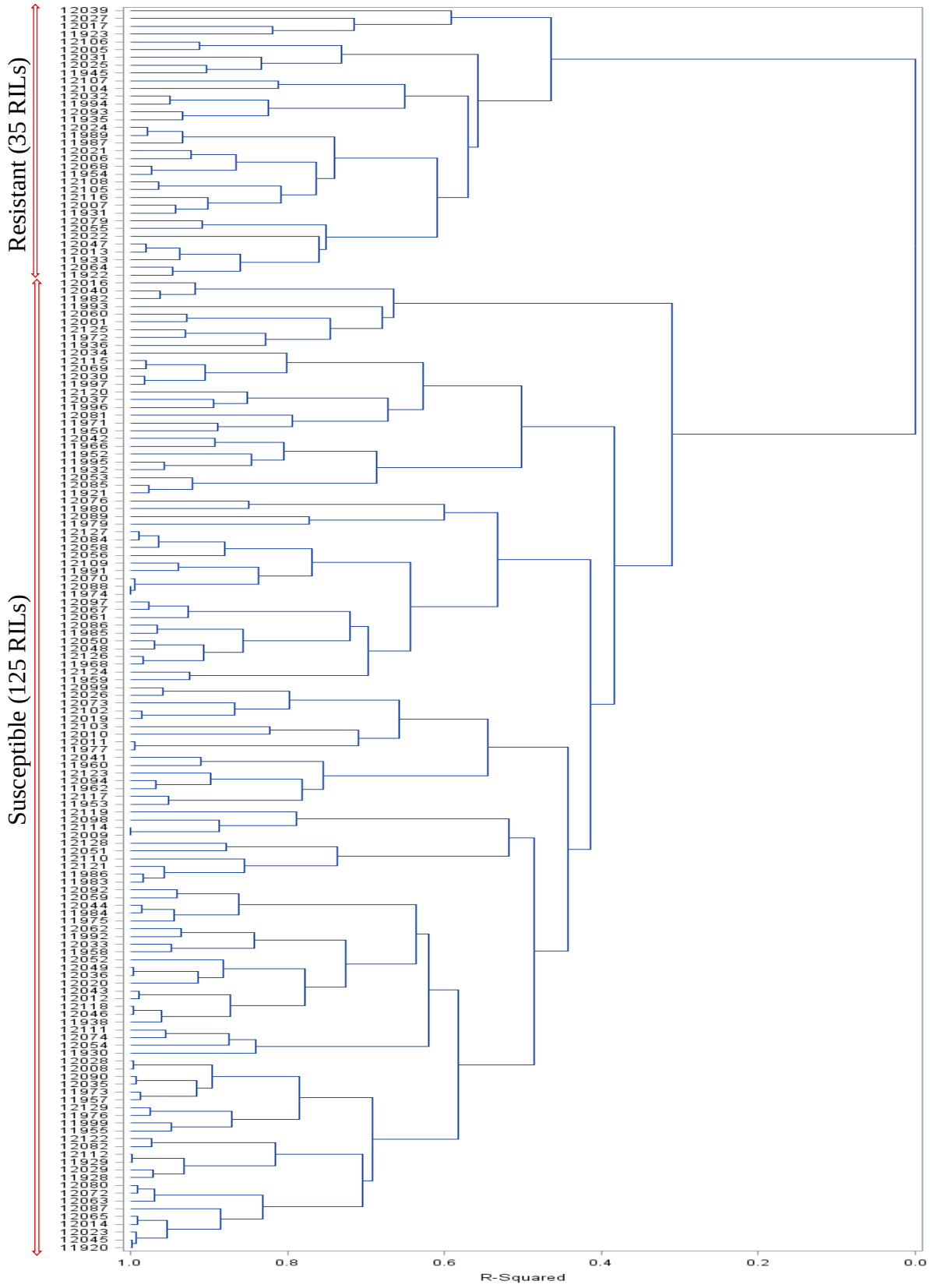


Figure 5.1 Dendrogram based on simple matching coefficients of flax wilt disease severity of 160 flax recombinant inbred lines derived from a cross of Aurore (wilt resistant) and Oliver (wilt susceptible) scored in wilt nurseries at seedling, early and late flowering stages and under controlled conditions at 14, 21 and 28 days after seeding, using *Fol* Isolates 65, 81 and 131.

5.4.2 Heritability of Resistance to Flax Wilt

High heritability for resistance to flax wilt was observed in the AO RIL population, while the broad sense heritability observed under controlled conditions for single isolate testing at 21 dai was moderate (Table 5.2).

Table 5.2 Genotypic (V_g), environmental (V_e) and phenotypic (V_p) variation and broad sense heritability (H^2) of flax wilt resistance in the Aurore x Oliver flax RIL population, at Saskatoon and Morden field wilt nurseries based on disease severity at late flowering stage, and single isolate testing with Isolates 65, 81 and 131 at 21 days after inoculation under controlled environment, 21 days after inoculation.

Trait	V_g	V_e	V_p	H^2
Disease severity at late flowering stage	4.036	0.463	4.499	0.897
Disease severity at 21 days after inoculation	3.459	3.132	6.591	0.525

5.5 Discussion

Wilt caused by the *Fol* pathogen is one of the most economically important diseases of flax world-wide. While chemical and cultural control methods can be utilized to reduce the yield and quality losses due to this disease, the most effective control is the cultivation of wilt resistant flax cultivars combined with integrated pest management (IPM) techniques. Thus, the selection for wilt resistant cultivars is a priority in flax breeding programs. One important aspect in this process is understanding the inheritance of flax wilt resistance. Two hypotheses have been suggested to explain the inheritance pattern of flax wilt resistance: polygenic resistance conferred by many minor genes and major gene resistance conferred by one or two genes, with additive effects from minor genes.

Above average flax wilt resistance was observed in accessions from North America, South America and East Asia, in a study conducted in Russia that evaluated 153 flax accessions from different parts of the world (Diederichsen et al., 2008). A high level of variation in disease severity was also observed among the accessions, with disease severity varying from 0% to 100% (Diederichsen and Fu, 2008). This variation is probably due to

the selection for wilt resistance over the years, with some of the early established cultivars loosing resistance over the years due to changes in the pathogen population or varied response of cultivars at different geographical locations (Kommedahl, et al., 1970).

The F_8 RIL population, used in this study was developed from the French cultivars: Aurore (spring, fibre type, resistant to fusarium wilt) and Oliver (winter, linseed type, susceptible to fusarium wilt). The inheritance of flax wilt resistance for the RIL population was in accordance with a two gene model, complying with the expected 3:1 ratio in a chi square test for Isolates 81 and 131 at 21 dai in the controlled environment and as well as early flowering stage in the Saskatoon wilt nursery. An inheritance pattern of 3:1 was also observed in a cluster analysis, which included all the wilt ratings from the controlled environment and the wilt nurseries. Based on the observations, flax wilt resistance in this population was conferred by recessive alleles of two independent genes, with the additive effects of minor genes modifying the resistant response (Fig 5.2).

Generation	**Probable Genotype**			
P (Homozygous)	AABB x aabb			
F1	AaBb			
F8	AABB 25%	AAbb 25%	aaBB 25%	aabb 25%
	Susceptible 3	:	Resistance 1	
P (Heterozygous)	AaBb x aabb			
F1	AaBb	aaBb	Aabb	aabb
F8	AABB 25%	AAbb 25%	aaBB 25%	aabb 25%
	Susceptible 3	:	Resistance 1	

Fig 5.2 Schematic representation explaining the 3:1 segregation of two independent genes conferring resistance to flax wilt in the cross Aurore x Oliver. The dominant alleles of the genes are represented by A and B, while the recessive form is represented as a and b.

Flax wilt resistance due to two genes was reported by Knowles and Houston (1955) with a highly wilt resistant selection, Dakota 48-94. They attributed the phenotypic observations based on disease severity, varying from resistant, moderately resistant, moderately susceptible and susceptible, to a 1:4:4:7 ratio, conferred by two major genes; Fu_A and Fu_B. A third gene Fu_c was identified with another clone (isolate) of the pathogen, and they proposed three major genes conferred flax wilt resistance in Dakota 48-94 along with effects from minor genes.

Two independent genes conferring fusarium wilt resistance in a DH flax population was suggested by Spielmeyer *et al*. (1998b) based on glasshouse and field experiments. They had considered three phenotype classes as resistant, intermediate and susceptible fitting a 1:2:1 ratio. However, they also observed DH lines with higher resistance than the resistant parent, an indication of additive effects of minor genes. Two quantitative loci of this population were mapped to linkage groups 6 and 10 in an amplified fragment length polymorphism (AFLP) map, which complied with the hypothesis of two major genes conditioning flax wilt resistance (Spielmeyer *et al*.,1998b).

In China, Bo et al. (2003), determined two dominant genes conferred resistant to flax wilt in the cultivar ‘Jin Ya 7’. These resistance genes were found to be closely linked with three specific bands observed in an AFLP analysis (as cited in Wu et al., 2015). These AG/CAG segments have been sequenced and transformed into a sequence characterized amplified region (SCAR) marker and are used in MAS in flax breeding.

Controlled conditions in a glasshouse or growth chamber are beneficial in determining the genetic effects of a disease, since the environmental conditions, soil microflora and pathogen inoculum concentration can be controlled. The complexity of *Fol* populations has

been suggested to hinder the identification of major gene resistance of flax wilt due to the presence of large number of races and the unpredictability of infection in the field as a result of varying conditions on different site years (Spielmeyer *et al*.,1998b). One disadvantage of major gene resistance is that it is race specific. Thus in the presence of a mixture of pathotypes or at different geographical locations with different pathotypes, major resistance may often be defeated and the polygenic resistance due to the underlying minor genes will determine the resistance response. Use of a homozygous population such as a RIL or DH population is useful in this situation, as these types of populations exclude the heterogeneity among the individuals conveying varying degrees of genetic resistance.

While digenic resistance was observed in this study and in a previous study by Spielmeyer et al. (1998b), the inheritance type or the gene expression was different. In the present study, complementary action of two independent genes was observed, while in their study incomplete dominance was observed. This was probably due to the different genetic backgrounds of the parents and different pathotypes of the pathogens used.

Inheritance patterns varied in two populations in the F_2 generation developed by crossing the same fusarium wilt resistant pigeon pea line with two susceptible pigeon pea lines (Ajay et al., 2013). The two populations showed different segregation patterns with one population expressing complementary gene action with a 9:7 ratio, and the other population displaying inhibitory gene action with a 13:3 ratio. The susceptible parents shared one dominant gene, with resistance to fusarium wilt in pigeon pea concluded to be governed by two or more genes in their recessive form. In a common bean (*Phaseolus vulgaris*) RIL population, three segregation patterns (1:1, 3:1 and 5:3) were observed for resistance to 11 races of *Colletotrichum lindemuthianum* causing anthracnose (Campa et al., 2014).

Broad sense heritability of 89.7% was observed with the RILs in three site years in this study, while under controlled conditions it was 55.5%. This difference is probably due to high variation of disease severity observed under controlled environment and considering the combination of three isolates significantly different from each other for the calculation of broad sense heritability (Chapter 3). Therefore, phenotypic evaluation for selection of flax wilt resistance within this population was reliable, since the environmental effect was small. Genotype x environment interactions affect the reliability of using the phenotype for cultivar selections (Perchepied and Pitrat, 2004). Therefore, this information would be useful in flax breeding programs to select and develop flax lines with effective resistance to flax wilt.

Mapping a population and analyzing the QTLs can be beneficial to locate the loci governing traits of origin within a genome. This can be used in MAS to reduce the time and labour of continuous selective breeding, although phenotype selection is essential for verification of genotype expression in the field. Based on this study, by mapping the two genes and the underlying loci conferring flax wilt resistance, the wilt resistance breeding process can be accelerated and can be used to develop even more resistant cultivars through gene pyramiding.

Chapter 6: General Discussion

Flax wilt caused by the facultative fungal saprophyte *Fol* is one of the major diseases affecting flax production and yield. The pathogen colonizes the xylem by entering the plant through the root or from infected seeds, obstructing water movement, thereby causing the upper parts of the plant to wilt. Due to the ubiquitous distribution of *Fol*, the presence of numerous pathotypes, and the ability to survive in the field for long periods in the absence of flax, it is a difficult disease to control in the Canadian Prairies (Kommedahl et al., 1970). Therefore, cultivation of flax wilt resistant cultivars is recommended and required for efficient control.

There are many pathotypes of *Fol*, which vary in their level of aggressiveness and geographic distribution (Baayen et al., 2000, Saharan et al., 2005). Therefore, phenotyping flax genotypes for wilt under controlled conditions, using a limited number of pathotypes can be useful to identify genetic resistance to the disease. In this study, the hypothesis was that the resistance to flax wilt is conferred by a dominant gene consistent with gene for gene theory. To accomplish that, first a controlled environment phenotyping method to screen flax wilt, was optimized using four flax cultivars and 17 *Fol* isolates (Chapter 3). We observed that even isolates collected from the same location, expressed different levels of aggressiveness, indicating the possible existence of races. However, inoculating a mixture of several isolates would simulate the pathogen structure similar to that which occurs in the field.

Three isolates were selected to screen a subset of 160 lines from a RIL population developed by crossing two cultivars used in the initial disease screening; Aurore and Oliver, under controlled conditions. Isolate selection was based on different disease reactions observed in resistant and susceptible cultivars, rate of disease progression, sporulation in MS medium and the location of origin of the isolate. The same RIL population was

phenotyped at two wilt nurseries over three site years (Chapter 4). Gradual disease development (with respect to disease severity at final rating and AUDPC for both environments) without a clear separation of resistant and susceptible lines, was observed indicating the involvement of many genes conferring wilt resistance. The isolates differed from each other, further indicating the existence of multiple pathotypes of *Fol* within a location and between geographical locations. The phenotypes of the RILs in the flax wilt field nursery were correlated with the phenotypes observed under controlled conditions in accordance with previous studies, thus verifying the benefits of wilt resistance phenotyping under controlled conditions as a predictor for cultivar performance in the field (Spielmeyer et al., 1998b, Kroes et al., 1998). Disease reaction under controlled conditions was more severe than was observed in the field, probably because optimum conditions were provided for disease development and due to lack of competition between isolates/ pathotypes for infection. RILs more resistant or susceptible than the two parents were observed, an indication of transgressive segregation. Therefore, in this AO RIL population minor genes also contribute to the wilt disease reaction observed, apart from possible major genes. However, repeating the field experiments at more locations and multiple years would provide more information about the population.

Resistance to flax wilt in this population was determined to be conferred by two independent recessive genes, based on the wilt nursery screening data (Chapter 5). Flax wilt susceptible parent, Oliver, could have been either homozygous dominant or heterozygous, while Aurore (wilt resistant parent) was probably homozygous recessive. However, the additive effects of minor genes also have contributed to the flax wilt reaction of the AO population, especially observable with transgressive segregation in wilt nurseries. The occurrence and competition of multiple pathotypes of *Fol* and different pathogens in the soil, and the segregation of multiple genes from parents with diverse

genetic backgrounds promoted the expression of minor genes, making it difficult to observe major gene resistance against *Fol* .

Segregation for resistance to flax wilt has been debated and two hypotheses exist: polygenic (Kommedahl et al., 1970), two independent genes with additive effects from minor genes (Spielmeyer et al., 1998b), and three major genes with minor genes altering the disease response (Knowles et al., 1956). The multiple hypotheses are most likely due to the differences in the cultivars, population types (DH, RIL, NIL), generation of the population (F_1, F_2, F_8), isolate or isolate mixtures and experimental conditions (field or controlled conditions). Based on the current study, as well as previous studies, it was concluded that resistance to flax wilt is conferred by major genes expressing race specific resistance consistent with gene for gene theory,, though observed only under optimal, controlled conditions. In the field, expression of minor genes may alter the disease response, providing race non-specific resistance.

Flax wilt resistance was inherited through two major genes with additive effects from minor genes. Therefore, it is necessary to identify the specific locations of these genes and QTL, to develop cultivars resistant to flax wilt. In addition, this information can be used to pyramid the wilt resistance genes to develop cultivars resistant to a wide range of pathotypes of *Fol*. The information can be used to select lines carrying flax wilt resistance genes through MAS, and increase the efficiency of disease resistant cultivar development in flax breeding programs.

Gene for gene theory states that for every avirulence gene in the pathogen that is capable of causing a disease, there is a resistant gene in the host conferring resistance against the pathogen. The interaction of an avirulence gene/ gene product and resistant gene/ gene product results in an incompatible reaction, thus conferring resistance. Based on the observations in the current study, it can be concluded that part of the hypothesis can be

accepted, that the resistance to flax wilt is conferred by two independent, recessive genes instead of one dominant gene, but consistent with the gene for gene theory.

Literature Cited

Agarwal, M., Shrivastava, N., & Padh, H. (2008). Advances in molecular marker techniques and their applications in plant sciences. *Plant Cell Rep, 27*, 617–631.

Agriculture and Agri-Food Canada, 2011. *Crop Identification and BBCH Staging Manual: SMAP-12 Field Campaign.* Retrieved May 2016 from https://smapvex12.espaceweb.usherbrooke.ca/BBCH_STAGING_MANUAL_GENERAL_ALL_CROPS.pdf

Ajay, B., Prasad, P., Byre Gowda, M., Ganapathy, K., Ganesh, B., Abdul Fiyaz, R., Veerakumar, G.; Prashanth Babu, H.; Venkatesha, S. & Ramya, K. (2013). Inheritance of resistance to Bangalore race of Fusarium wilt disease in pigeonpea (Cajanus cajan L.). *Australian Journal of Crop Science, 7*(10), 1520-1524.

Alabouvette, C., & Couteaudier, Y. (1992). Biological control of Fusarium wilts with nonpathogenic fusaria. *Biological Control of Plant Diseases, 230*, 415-426.

Alheit, K., Reif, J., Maurer, H., Hahn, V., Weissmann, E., Miedaner, T., & Wurschum, T. (2011). Detection of segregation distortion loci in triticale (x Triticosecale Wittmack) based on a high-density DArT marker consensus genetic linkage map. *BMC Genomics, 12*, 578. doi:10.1186/1471-2164-12-380

Allaby, R. G., Peterson, G. W., & David, A. (2005). Evidence of the domestication history of flax (Linum usitatissimum L.). *Theor Appl Genet, 112*, 58–65.

Amin, T., & Thakur, M. (2014). Linum usitatissimum L. (Flaxseed)–A Multifarious Functional Fo. *Online International Interdisciplinary Research Journal, IV*(I), 220-238.

Annonymous. (2012, December 13). *Linola headed for deregistration.* Retrieved November 2015, from http://www.agcanada.com/daily/linola-headed-for-deregistration

Arny, A. C. (1943). Flax Varieties Registered, I. *Agronomy, 35*(9), 823-824.

Arvayo-Ortiz, R., Esqueda, M., Acedo-Felix, E., Sanchez, A., & Gutierrez, A. (2011). Morphological variability and rces of Fusaium xysporum f. sp. ciceris associated with chickpea (Cicer arietinum) crops. *American Journal of Agriculture and Biological Sciences, 6*(1), 114-121.

Assigbetse, K., Fernandez, D., Dubois, M., & Geiger , J.-P. (1994). Differentiation of Fusarium oxysporum f. sp. vasinfectum Races on Cotton. *Phytopathology, 84*(6), 622-626.

Azmach, G., Gedil, M., Menkir, A. & Spillane, C. (2013). Marker-trait association analysis of functional gene markers for provitamin A levels across diverse tropical yellow maize inbred lines. BMC Plant Biol, 13(227).

Baayen, R., O'Donnell, K., Bonants, P., Cigelnik, E., Kroon, L., Roebroeck, E., & Waalwijk, C. (2000). Gene genealogies and AFLP analyses in the Fusarium oxysporum complex identify monophyletic and nonmonophyletic *formae speciales* causing wilt and rot disease. *Phytopathology, 90*(8), 891-900.

Bansal, V., Tewhey, R., LeProust, E., & Schork, N. (2011). Efficient and Cost Effective Population Resequencing by Pooling and In-Solution Hybridization. *PLoS ONE, 6*(3). doi:10.1371/journal.pone.0018353

Bodénès, C., Chancerel, E., Ehrenmann, F., Kremer, A., & Plomion, C. (2016). High-density linkage mapping and distribution of segregation distortion regions in the oak genome. *DNA Research*. doi:10.1093/dnares/dsw001

Booker, H., & Lamb, E. (2012). Quantification of low-level GM seed presence in Canadian commercial flax stocks. *AgBioForum, 15*(1), 31-35.

Booker, H., Mischkolz, J., St. Louis, M., & Lamb, E. (2014). Analysis of the Prevalence of CDC Triffid Transgenic Flax in Canadian Grain Stocks. *AgBioForum, 17*(1), 75-83.

Bradshaw Jr., H., Otto, K., Barbara, E., McKay, J., & Schemske, D. (1998). Quantitative Trait Loci Affecting Differences in Floral Morphology Between Two Species of Monkeyflower (Mimulus). *Genetics, 149*, 367-382.

Broman, K. (2005). The Genomes of Recombinant Inbred Lines. *Genetics, 169*(2), 1133–1146.

Business Insights: Essentials. (2014, Feb. 5). *Health Canada OKs cholesterol lowering claim for flax.* Retrieved from NewHope360.com : http://bi.galegroup.com.cyber.usask.ca/essentials/article/GALE%7CA357774294/2134edd0efc6fc3123b044ab881a4315?u=usaskmain

Campa, A., Rodríguez-Suárez, C., Giraldez, R., & José Ferreira, J. (2014). Genetic analysis of the response to eleven Colletotrichum lindemuthianum races in a RIL population of common bean (Phaseolus vulgaris L.). *BMC Plant Biology, 14*(115). doi:10.1186/1471-2229-14-115

Chikh-Rouhou, H., González-Torres, R., Oumouloud, A., & Alvarez, J. (2011). Inheritance of race 1.2 Fusarium wilt resistance in four melon cultivars. *Euphytica, 182*, 177-186.

Chittem, K., & Kulkarni, S. (2008). Effect of Media on the Growth of Fusarium oxysporum f. sp. gerberae and Fusarium oxysporum f. sp dianthi. *Karnataka J. Agric. Sci., 21* (2), 303-304.

Climate, G. o. (n.d.). Retrieved 06 17, 2015, from http://climate.weather.gc.ca/climate_normals/index_e.html#1981

Cloutier, S., Ragupathy, R., Miranda, E., Radovanovic, N., Reimer, E., Walichnowski, A., Ward, K.; Rowland, G.; Duguid, S. & Banik, M. (2012). Integrated consensus genetic and physical maps of flax (Linum usitatissimum L.). *Theoretical and Applied Genetics, 125*, 1783-1795. doi:DOI 10.1007/s00122-012-1953-0

Cloutier, S., Ragupathy, R., Niu, Z., & Duguid, S. (2011). SSR-based linkage map of flax (Linum usitatissimum L.) and mapping of QTLs underlying fatty acid composition traits. *Molecular Breeding, 28*(4), 437-451.

Conner, J. K. & Hartl, D. L., (2004). Quantitative genetics I: Genetic variation. In: A primer of ecological genetics. s.l.:Sunderland, Mass. : Sinauer Associates, 97-136.

Cullis, C. (2005). Mechanisms and Control of Rapid Genomic Changes in Flax. *Annals of Botany, 95*, 201–206.

Cullis, C. A. (2007). Flax. In C. Kole (Ed.), *Oilseeds* (Vol. 2).

Diederichsen, A., & Fu, Y.-B. (2008). Flax Genetic Diversity as the Raw Material for Future Success. *International Conference on Flax and Other Bast Plants*, 21-23.

Diederichsen, A., Rozhmina, T., & Kudrjavceva, L. (2008). Variation patterns within 153 flax (Linum usitatissimum/ L.) genebank accessions based on evaluation for resistance to fusarium wilt, anthracnose and pasmo. *Plant Genetic Resources: Characterization and Utilization, 6*(01), 22-32.

Dodds, P., Lawrence, G., Catanzariti, A., Teh, T., Wan, C., Ayliffe, M., Kobe, B. & Ellis, J. (2006). Direct protein interaction underlies gene-for-gene specificity and coevolution of the flax resistance genes and flax rust avirulence genes. *Biological Sciences - Plant Biology, 103*(23), 8888-8893.

Dribnenkil , J., & Green, A. (1995). Linola TM '947' low linolenic acid flax. *Can. J. Plant Sci., 75*, 201-202.

Duguid, S. (2009). Flax. In J. Vollmann, & I. Rajcan, *Oil Crops. Series: Handbook of Plant Breeding* (Vol. 4, pp. 233-256). doi:10.1007/978-0-387-77594-4

Duguid, S. S. (2010). Flax. In J. Vollmann, & I. Rajcan, *Oil Crops* (pp. 233-255).

Elias, K., & Schneider, R. (1991). Vegetative compatibility groups in Fusarium oxysporum f. sp. lycopersici. *Phytopathology, 81*(2), 159-162.

FAOSTAT. (2014). *http://faostat3.fao.org/faostat-gateway/go/to/browse/Q/QC/E*. Retrieved September 2014, from FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS.

Fikere, M., Tadesse, T., Gebeyehu , S., & Hundie , B. (2010). Agronomic performances, disease reaction and yield stability of field pea (Pisum sativum L.) genotypes in Bale Highlands, Ethiopia. *Australian Journal of Crop Science, 4*(4), 238-246.

Flax Council of Canada. (2015). *Introduction*. Retrieved August 2015, from http://flaxcouncil.ca/growing-flax/introduction/

Flax Council of Canada. (2015). *Varieties*. Retrieved August 2015, from Flax Council of Canada: http://flaxcouncil.ca/growing-flax/varieties/

Flax. (n.d.). Retrieved from http://www.flaxcouncil.ca/english/index.jsp?p=what2&mp=what

FlaxStatistics. (2014). *http://www.flaxcouncil.ca/english/index.jsp?p=statistics2&mp=statistics*. Retrieved September 2014, from Flax Council of Canada.

Flor, H. (1953). Wilt, Rust and Pasmo of Flax. *The Yearbook of Agriculture*, 869-873. Retrieved from http://naldc.nal.usda.gov/download/IND43894445/PDF

Flor, H. (1965). Tests for allelism of rust-resistance genes in flax. *Crop Science, 5*, 415-418.

Foster, R., Pooni, H., & MacKay, I. (1997). Quantitative evaluation of Linum usitatissimum varieties for dual-purpose traits. *The Journal of Agricultural Science, 129*(2), 179--185.

Fu, Y.-B., Rowland, G., Duguid, S., & Richards, K. (2003). RAPD analysis of 54 North American flax cultivars. *Crop Science, 43*(4), 1510-1515.

Fuchs, J.-G., Moënne-Loccoz, Y., & Défago, G. (1997). Nonpathogenic Fusarium oxysporum Strain Fo47 Induces Resistance to Fusarium Wilt in Tomato. *Plant Disease, 81*(5), 492-496.

Gowda, S., Radhika, P., Kadoo, N., Mhase, L. B., & Gupta, V. (2009). Molecular mapping of wilt resistance genes in chickpea. *Mol Breeding , 24*, 177–183.

Green, A., & Marshall, D. (1984). Isolation of induced mutants in linseed (Linum usitatissimum) having reduced linolenic acid content. *Euphytica, 33*, 321-328.

Grisel , J. (2000). Quantitative trait locus analysis. *Alcohol Res Health, 24*(3), 169-74.

Harlan, J. (1971). Agricultural origins: centers and noncenters. *Science 174:468–474, 174*, 468–474.

Heller, K., Guan, F., Hua, L., Alexopoulou, E., Wu, G., Jankauskienė, Z., Fu, W. & Sheng, Q. (2014). A comparative study between Europe and China in crop management of two types of flax: linseed and fibre flax. *Ind. Crops Prod.* doi:http://dx.doi.org/10.1016/j.indcrop.2014.07.010

Holland, J. B., Nyquist, W. E. & Cervantes-Martín, C. T., (2003). Estimating and interpreting heritability for plant breeding: an update. *Plant breeding review, 22*, 9-112..

Hong, E., & Park, J. (2012). Sample Size and Statistical Power Calculation in Genetic Association Studies. *Genomics Inf. , 10*(2), 117–122.

Hoper, H., Steinberg, C., & Alabouvette, C. (1995). Involvement of Clay Type and pH in the Mechanisms of Soil Suppressiveness to Fusarium Wilt of Flax. *Soil Biol Biochem., 27*(7), 955-967.

Houston, B., & Knowles, P. (1949). Fifty-year survival of flax Fusarium wilt in the absence of flax culture. *Plant Dis., 33* , 38-39.

Islam, M., & Mayo, G. (1990). A Compendium on Host Genes in Flax Conferring Resistance to Flax Rust. *Plant Breeding, 104*(2), 89–100.

Islam, R. (2015). Variability of Growth and Sporulation of Fusarium Soil Isolates on Different Culture Media. *International Journal of Research , 2*(2), 160-167.

Jhala, A., & Hall, L. (2010). Flax (Linum usitatissimum L.): Current Uses and Future Applications. *Australian Journal of basic and Applied Sciences, 4*(9), 4304-4312.

Jiménez-Fernández, D., Landa, B. B., Kang, S., Jiménez-Díaz, R., & Navas-Cortés, J. (2013). Quantitative and Microscopic Assessment of Compatible and Incompatible Interactions between Chickpea Cultivars and Fusarium oxysporum f. sp. ciceris Races. *PLoS One, 8*(4). doi:10.1371/journal.pone.0061360

Joshi, R., & Nayak, S. (2010). Gene pyramiding-A broad spectrum technique for developing durable stress resistance in crops. *Biotechnology and Molecular Biology Review, 5*(3), p. 51-.

Kislev , M. E., Simchoni , O., Melamed , Y., & Maroz, L. (2011). Flax seed production: evidence from the early Iron Age site. *Veget Hist Archaeobot, 20*, 579–584.

Knowles, P., & Houston, B. (1955). Inheritance of Resistance to Fusarium Wilt of Flax in Dakota Selection 48-94. *Agronomy*, *47*(3), 131-135.

Knowles, P., Houston, B., & McOnie, J. (1956). Inheritance of Resistance to Fusarium Wilt of Flax in Punjab 53. *Agronomy, 48*(3), 135-137.

Kommedahl, T., Christensen, J., & Frederiksen, R. (1970). A Half Century of Research in Minnesota on Flax Wilt Caused by Fusarium Oxyporum. *Agricultural Experiment Station, Technical Bulletin*.

Korte, A., & Farlow, A. (2013). The advantages and limitations of trait analysis with GWAS: a review. *Plant Methods*, *9*(29). Retrieved from http://www.plantmethods.com/content/9/1/29

Kroes, G., Baayen, R., & Lange, W. (1998). Histology of root rot of flax seedlings (Linum usitatissimum) infected by Fusarium oxysporum f.sp. lini. *European Journal of Plant Pathology*, *104*(7), 725-736.

Kroes, G., Sommers, E., & Lange, W. (1998). Two in vitro assays to evaluate resistance in Linum usitatissimum to Fusarium wilt disease. *European Journal of Plant Pathology, 104*, 561-568.

Kroes, I. (1997). Aspects of resistance of flax and linseed (Linum usitassimum) to Fusarium oxysporum f. sp. lini. In *Fusarium resistance in flax and linseed across locations and years* (pp. 105-135).

Kroes, I., Loffler, H., Parlevliet, J., & Langa, W. (1997). Do races in Fusarium oxysporum f. sp lini exist? In *Aspects of resistance of flax and linseed (Linum usitatissimum) to Fusarium oxysporum f. sp. lini* (pp. 91-103).

Kumar, S., Banks, T., & Cloutier, S. (2012b). Review Article : SNP Discovery through Next-Generation Sequencing and its Applications. *International Journal of Plant Genomics, 2012*. doi:10.1155/2012/831460

Kumar, S., You, F., & Cloutier, S. (2012). Genome wide SNP discovery in flax through next generation sequencing of reduced representation libraries. *BMC Genomics, 13*, 684. doi:10.1186/1471-2164-13-684

Kvavadze, E., Bar-Yosef, O., Belfer-Cohen, A., Boaretto, E., Jakeli, N., Matskevich, Z., & Meshveliani, T. (2009, SEPTEMBER 11). 30,000-Year-Old Wild Flax Fibers. *SCIENCE, 325* , 1359.

Lamb, E., & Booker, H. (2011). Quantification of low-level genetically modi ed (GM) seed presence in large seed lots: a case study of GM seed in Canadian flax breeder seed lots. *Seed Science Research, 21*(4), 315-321.

Leslie, J. (1993). Fungal Vegetative Compatibility. *Annual review of phytopathology , 31*(1), 127-150.

Li, C., Bai, G., Chao, S., & Wang, Z. (2015). A High-Density SNP and SSR Consensus Map Reveals Segregation Distortion Regions in Wheat. *Biomed Res Int. 2015; 2015: 830618*. doi:10.1155/2015/830618

Li, M., Shi, J., Xie, X., Leng, Y., Wang, H., Xi, P., Zhou, J.; Zhong, S. & Jiang, Z. (2013). Identification and application of a unique genetic locus in. *Canadian Journal of Phytopathology*. doi:10.1080/07060661.2013.828321

Liu, X., Guo, L., You, J., Liu, X., He, Y., Yuan, J., Liu, G. & Feng, Z. (2010). Progress of Segregation Distortion in Genetic Mapping of Plants. *Research Journal of Agronomy, 4*(4), 78-83.

Lochhead, A., & Cook, F. (1961). Microbial Growth Factors in Relation to Resistance of Flax Varieties to Fusarium Wilt. *Canadian Journal of Botany, 39*, 7-18.

Lorieux, M., Perrier, X., Goffinet, B., Lanaud, C., & Gonzalez de Leon, D. (1995). Maximum-likelihood models for mapping genetic markers showing segregation distortion. 2. F2 populations. *Theor. Appl. Genet., 90*, 81-89.

Ma, L.-J., Geiser, D., Proctor, R., Rooney, A., O'Donnell, K., Trail, F., Gardiner, D.; Manners, J. & Kazan, K. (2013). Fusarium pathogenomics. *Annual review of microbiology, 67*, 399-416.

Marcel, T., Gorguet, B., Ta, M., Kohutova, Z., Vels , A., & Niks , R. (2008). Isolate Specificity of Quantitative Trait Loci for Partial Resistance of Barley to Puccinia hordei. *The New Phytologist*, *177*(3), 743-755.

Marshall, G. (1992). Recent developments in flax breeding relevant to production technologies. *Industrial Crops and Products, 1*(2-4), 273-281.

Martin, T. P. (2001). Observations of Mycorrhizal Inoculation of Pin and Scarlet Oak. Thesis submitted to the Faculty of the Virginia Polytechnic Institute.

McClean, P. (1998). *Mapping quantitative loci.* Retrieved December 2015, from https://www.ndsu.edu/pubweb/~mcclean/plsc731/quant/quant1.htm

McHughen, A., Rowland, G., Holm, F., Bhatty, R., & Kenaschuk, E. (1997). CDC Triffid transgenic flax. *Canadian Journal of Plant Science, 77*, 641–643.

Miles, C., & Wayne, M. (2008). Quantitative Trait Locus (QTL) Analysis. *Nature Education, 1*(1), 208. Retrieved from http://www.nature.com/scitable/topicpage/quantitative-trait-locus-qtl-analysis-53904

Mina , U., & Dubey, S. (2010). Effect of environmental variables on development of Fusarium wilt in chickpea (Cicer arietinum) cultivars. *Indian Journal of Agricultural Sciences, 80*(3), 233-236.

Mittapalli, O., & Rowland, G. (2003). Inheritance of seed color in flax. *Crop Science, 43*, 1945-1951.

Mpofu, S., & Rashid, K. (2001). Vegetative Compatibility GroupsWIthin Fusarium oxysporum f. sp. lini fron Linum usitatissimum (flax) Wilt Nurseries in Western Canada. *Canadian journal of Botany, 79*, 836-843.

Muir, A., & Westcott, N. (2003). Current regulatory status of flaxseed and commercial products. In A. D. Muir, & N. D. Westcott, *Flax : The genus Linum* (pp. 292 - 297)..

Musoni, A., Kimani, P., Narla, R., Buruchara, R., & Kelly, J. (2010). Inheritance of fusarium wilts (Fusarium oxysporum f. sp. phaseoli) resistance in climbing beans . *African Journal of Agricultural Research, 5*(5), 399-404.

Olaniyi, O. O., Kehinde, O. B., Oduwaye, O. A., Adenuga, O., Mapayi , E., & Adepoju, A. (2013). Genotype Main Effect and Genotype x Environment (GGE Bi-Plot) Model of Multi-Environmental Trial of Melon (Citrullus lanatus). *Research Journal of Applied Sciences, Engineering and Technology, 06*(02), 223-227.

Oomah, B., & Mazza, G. (1999). Health benefits of phytochemicals from selected Canadian crops. *Trends in Food Science & Technology , 10*, 193-198.

Oplinger E.S., O. E. (n.d.).

Oumouloud, A., El-Otmani, M., Chikh-Rouhou, H., Claver, A., Torres, R., Perl-Treves, R., & Álvarez, J. (2013). Breeding melon for resistance to Fusarium wilt: recent developments. *Euphytica, 192*(2), 155-169.

Peleman, J., Wye, C., Zethof, J., Sorensen, A., Verbakel, H., Van Overen, J., Gerats, T. & Ven der Voort, J. (2005). Quantitative Trait Locus (QTL) Isogenic Recombinant Analysis: A Method for High-Resolution Mapping of QTL Within a Single Population. *Genetics, 171*, 1341-1352.

Pengilly, N. (2005). *The Essential Flax: A compendium of Diet Reference, Information, Facts, Folklore, Recipes and Research.* Saskatchewan Flax Development Commission.

Perchepied, L., & Pitrat , M. (2004). Polygenic Inheritance of partial resistance to Fusarium oxysporum f. sp. melonis race 1.2 in melon. *Phytopathology, 94*(12), 1331-1336.

Perchepied, L., Dogimont, C., & Pitrat, M. (2005). Strain-specific and recessive QTLs involved in the control of partial resistance to Fusarium oxysporum f. sp. melonis race 1.2 in a recombinant inbred line population of melon. *Theoretical and applied genetics, 111*(1), 65-74.

Przybylski, R. (2005). Flax oil and high linolenic oils. *Bailey's industrial oil and fat products*.

Pu, Z., Shimizu, M., Zhang, Y., Nagaoka, T., Hayashi, T., Hori, H., Matsumoto, S.; Fujimoto, R. & Okazaki, K. (2012). Genetic mapping of a fusarium wilt resistance gene in Brassica oleracea. *Molecular Breeding, 30*(2), 809-818.

Puhalla, J. (1985). Classification of strains of Fusarium oxysporum on the basis of vegetative compatibility. *Canadian Journal of Botany, 63*, 179-183.

Rashid, K. (2003). Principal Diseases of Flax. In *Flax: Genus Linum* (pp. 92-123). Taylor and Francis Ltd, London.

Rashid, K. Y., & Kenaschuk, E. O. (1999). Three new rust resistance genes in flax introductions. *Canadian Journal of Plant Pathology, 21*(1), 64-69.

Rashid, K., Kutcher, H., Desjardins, M., & Duguid, S. (2014). Diseases of Flax in Manitoba and Saskatchewan in 2013. In *Canadian Plant Disease Survey* (Vol. 94, pp. 187-188).

Rogers, P., & Stevenson, W. (2010). Aggressiveness and Fungicide Sensitivity of Alternaria dauci. *Plant Disease, 94*(4), 405-412.

Rowland, G. (1991). An EMS-induced low-linolenic-acid mutant in McGregor flax. *Can. J. Plant Sc. , 71*, 393-396.

Rowland, G. (n.d.). *Flax*. Retrieved December 11, 2014, from The Encyclopedia of Saskatchewan: http://esask.uregina.ca/entry/flax.html

Rutkowska-Krause, L., Mankowska, G., Lukaszewicz, M., & Szopa , J. (2003). Regeneration of flax (Linum usitatissimum L.) plants from anther culture and somatic tissue with increased resistance to Fusarium oxysporum. *Plant Cell Rep, 22*,110-116

Saharan, G., Mehta, N., & Sangwan, M. (2005). Fungal Diseases of Linseed. In G. S. Saharan, N. Mehta, & M. S. Sangwan, *Diseases of Oilseed Crops* (pp. 176-201). Indus Publishing.

Sampling and testing protocol for Canadian flaxseed exported to the European Union. (2015, August). Retrieved from Canadian Grain Commission,: http://www.grainscanada.gc.ca/gmflax-lingm/stpf-peevl-eng.htm

Scher, F., & Baker, R. (1980). Mechanism of biological control in a Fusarium-suppressive soil. *Phytopathology, 70*(5), 412-417.

Sehgal, D., Singh, R., & Rajpal, V. (2016). Quantitative trait loci mapping in plants: Concepts and approaches. In *Molecular Breeding for Sustainable Crop Improvement* (pp. 31-59).

Semagn, K., Bjørnstad, A., & Ndjiondjop, M. (2006). Principles, requirements and prospects of genetic mapping in plants. *African Journal of Biotechnology, 5*(25), 2569-2587.

Semagn, K., Bjørnstad, Å., & Ndjiondjop, M. (2006b). Review: An overview of molecular marker methods for plants. *African Journal of Biotechnology, 5*(25), 2540-2568.

Serra-Wittling, C., Houot, S., & Alabouvette, C. (1996). Increased Soil Suppressiveness to Fusarium Wilt of Flax After Addition of Municipal Soil Waste Compost. *Soil Biol. Biochem, 28*(9), 1207-1214.

Sherbakoff, C. (1949). Breeding for resistance to Fusarium and Verticillium wilts. *The Botanical review, 15*(6), 395-399.

Silme, R., & Cagirgan, M. (2010). Screening for resistance to Fusarium wilt in induced mutants snf world collection of sesame under intensive management. *Turkish Journal of Field Crops, 15*(1), 89-93.

Sonah, H., O'Donoughue, L., Cober, E., Rajcan, I., & Belzile, F. (2014, November). Combining Genome-wide Association and QTL Analysis: Opportunities and Challenges. *ISB News Report*. Retrieved from http://www.isb.vt.edu/news/2014/Nov/SonahODonoughueCoberRajcanBelzile.pdf

Sonah, H., O'Donoughue, L., Cober, E., Rajcan, I., & Belzile, F. (2015). Identification of loci governing eight agronomic traits using a GBS-GWAS approach and validation by QTL mapping in soya bean. *Plant Biotechnol J, 13,* 211–221. doi:10.1111/pbi.12249

Sosulski, F., & Gore, R. (1964). The Effect of Photoperiod and Temperature on the Characteristics of Flaxseed Oil. *Canadian Journal of Plant Science, 44*, 382.

Soto-Cerda, B. J., Diederichsen, A., Ragupathy, R. & Cloutier, S., 2013. Genetic characterization of a core collection of flax (Linum usitatissimum L.) suitable for association mapping studies and evidence of divergent selection between fiber and linseed types. *BMC Plant Biol,* 13(78).

Sounigo, O., Lachenaud, P., Bastide, P., Cilas, C., Goran, J., & Lanaud, C. (2003). Assessment of the value of doubled haploids as progenitors. *Journal of Applies Genetics, 44*(3), 339-353.

Spielmeyer, W., Green, A., Bittisnich, D., Mendham, N., & Lagudah, E. (1998). Identification of quantitative trait loci contributing to Fusarium wilt resistance on an AFLP linkage map of flax (Linum usitatissimum). *Theor Appl Genet, 97*, 97: 633-641.

Spielmeyer, W., Lagudah, E., Mendham, N., & Green, A. (1998b). Inheritance of resistance to flax wilt (Fusarium oxysporum f.sp. lini Schlecht)in a doubled haploid population of Linum usitatissimum L. *Euphytica, 101*, 287-291.

Sun, J. (2015). *Flowering time in 5-azacytidine mutants of oilseed flax (Linum usitatissimum L.).* (MSc Thesis) Department of Plant Sciences, College of Agriculture and Bioresouces, University of Saskatchewan, Saskatoon, Saskatchewan.

Sun, S., & Huang, J. (1985). Formulated soil amendment for controlling Fusarium wilt and other soilborne diseases. *Plant disease*, *69*(11), 917-920.

Tekeoglu, M., Tullu, A., Kaiser, W., & Muehlbauer, F. (2000). Inheritance and Linkage of Two Genes that Confer Resistance to Fusarium Wilt in Chickpea. *Crop Sci., 40*, 1247-1251.

The Western Committee on Plant Diseases. (2012). Diseases of Oilseed Crops. In *Guidelines For The Control Of Plant Diseases In Western Canada* (p. 6). Retrieved from http://www.westernforum.org/Documents/WCPD/WCPD_documents/Current%20Guideline%20Files/Ch%204%20Diseases%20of%20Oilseeds.pdf

Ulloa, M., Hutmacher, R., Roberts, P., Wright, S., Nichols, R., & Davis, R. (2013). Inheritance and QTL mapping of Fusarium wilt race 4 resistance in cotton. *Theor. Appl. Genet., 126*, 1405-1418.

Vaisey-Genser, M., & Morris, D. (2003). Introduction: history of the cultivation and uses of flaxseed. In A. Muir, & N. Westcott, *Flax: The Genus Linum* (p. 7).

Varshney, R. K., Hoisington, D. A., Nayak, S. N. & Graner, A., 2009. Molecular Plant Breeding: Methodology and Achievements. In: *Methods in Molecular Biology, Plant Genomics.* s.l.:Humana Press, pp. 283-304.

Viju, C., Yeung, M., & Kerr, W. (2011). *Post-Moratorium EU Regulation of Genetically Modified Products: Triffid Flax.* Canadian Agricultural Trade Policy Research Network.

Visscher, P., Hill, W., & Wray, N. (2008). Heritability in the genomics era — concepts and misconceptions. *Nature Reviews Genetics, 9*(4), 255-266.

Wang, G. et al., 2014. Marker--trait association analysis of kernel hardness and related agronomic traits in a core collection of wheat lines. *Molecular Breeding,* 34(1), pp. 177-184.

Wang, Z., Hobson, N., Galindo, L., Zhu, S., Shi, D., McDill, J., Yang, L.; Hawkins, S.; Neutelings, G.; Datla, R.; Lambert, G.; Galbraith, D. W. ; Grassa, C. J. ; Geraldes, A.; Cronk, Q. C. ; Cullis, C. ; Dash, P. K. ; Kumar, P. A.; Cloutier, S.; Sharpe, A. G.; Wong, G. K.-S; Wang, J. & Deyholos, M. (2012). The genome of flax (Linum usitatissimum) assembled de novo from short shotgun sequence reads. *The Plant Journal, 72*, 461–473.

Wilson, I. (1946). Observations on wilt disease of flax. *Transactions of the British Mycological Societ, 29*(4), 221-231.

Wilson, I. (1946). Observations on Wilt Disease of Flax. *Transactions of the British Mycological Society, 29*(4), 221–231.

Wu, G., Yu, Y., Yuan, H., Wu, J., Liu, Y., Chen, S., Cheng, L.; Kang, Q.; Huang, W.; Xie, D.; Yao, Y.; Song, X.; Zhang, L.; Guang, F. & Heller, K. (2015). Advances in molecular

techniques used flax research in China. *Molecular Biology, 4*(4). doi:10.4172/2168-9547.

Xu, S. (2003). Theoretical Basis of the Beavis Effect. *Genetics, 165*(4), 2259–2268. Retrieved from www.genetics.org/content/genetics/165/4/2259.full.pdf

Xu, S. (2008). Quantitative Trait Locus Mapping Can Benefit From Segregation Distortion. *Genetics, 1*(4), 2201–2208.

Yamagashi, M., Takeuchi, Y., Tanaka, I., Kono, I., Murai, K., & Yano, M. (2010). Segregation distortion in F2 and doubled haploid populations of temperate japonica rice. *Journal of Genetics, 89*(2), 237-241.

You, F. M., Duguid, S. D., Thambugala, D., & Cloutier, S. (2013). Statistical analysis and field evaluation of the type 2 modified augmented design (MAD) in phenotyping of flax (Linum usitatissimum) germplasms in multiple environments. *American Journal of Crop Science, 7*(11), 1789-1800.

You, F.M., Song Q., Jia G., Cheng Y., Duguid S., Booker H., & Cloutier S. (2016). Estimation of genetic parameters and their sampling variances for quantitative traits in the type 2 modified augmented design, *The Crop Journal*. *4*(2), 107-118.doi: 10.1016/j.cj.2016.01.003

Young, L., Hammerlindl, J., Babic, V., McLeod, J., Sharpe, A., Matsalla, C., Bekkaoui, F.; Marquess, L. & Booker, H. (2015). Genetics, structure, and prevalence of FP967 (CDC Triffid) T-DNA in flax. *SpringerPlus*, *4*(146). doi:10.1186/s40064-015-0923-9

Zhang, S., Miao, H., Yang, Y., Xie, B., Wang, Y., & Gu, X. (2014). A major quantitative trait locus conferring resistance to fusarium wilt was detected in cucumber by using recombinant inbred lines. *Molecular Breeding*, *34*(4), 1805-1815.

Zhang, T. (2013). Characterizing the Flax Core Collection for Earliness. Thesis submitted to the Department of Plant Sciences, University of Saskatchewan.

Zohary, D., & Hopf, M. (2001). Oil and fiber crops. In *Domestication of Plants in the Old World: The Origin and Spread of Cultivated Plants in West Asia, Europe, and the Nile Valley* (pp. 126-132). Oxford University Press. Retrieved from http://www.myilibrary.com/?ID=81990

Zou, F., Gelfond, J., Airey, D., Lu, L., Manly, K., Williams, R., & Threadgil, D. (2005). Quantitative Trait Locus Analysis Using Recombinant Inbred Intercrosses:Theoretical and Empirical Considerations. *Genetics, 170,* 1299–1311.

Zou, G., Zhai, G., Feng, Q., Yan, S., Wang, A., Zhao, Q., Shao, J.; Zhang, Z.; Zou, J.; Han, B. & Tao, Y. (2012). Identification of QTLs for eight agronomically important traits using an ultra-high-density map based on SNPs generated from high-throughput sequencing in sorghum under contrasting photoperiods. *Journal of Experimental Botany*. doi:10.1093/jxb/ers205

Appendix I: Pairwise comparisons between cultivars based on Tukey's test for disease severity at 21 and 28 days after inoculation (dai) and area under disease progress curve (AUDPC).

Variable	Cultivars	Cultivars	Estimate	Standard Error	DF	t Value	Adjusted P
Disease severity at 21 dai	Aurore	Bison	0.1503	0.0758	132	1.98	0.1999
	Aurore	Novelty	-0.2229	0.0726	132	-3.07	0.0136
	Aurore	Oliver	-0.6648	0.0636	132	-10.46	<0.0001
	Bison	Novelty	-0.3732	0.0750	132	-4.97	<0.0001
	Bison	Oliver	-0.8151	0.0664	132	-12.28	<0.0001
	Novelty	Oliver	-0.4419	0.0627	132	-7.05	<0.0001
Disease severity at 28 dai	Aurore	Bison	0.1618	0.0549	132	2.95	0.0196
	Aurore	Novelty	-0.3578	0.0475	132	-7.54	<0.0001
	Aurore	Oliver	-0.5437	0.0451	132	-12.06	<0.0001
	Bison	Novelty	-0.5197	0.0508	132	-10.23	<0.0001
	Bison	Oliver	-0.7055	0.0486	132	-14.52	<0.0001
	Novelty	Oliver	-0.1858	0.0399	132	-4.65	<0.0001
AUDPC	Aurore	Bison	0.0761	0.0655	132	1.16	0.6522
	Aurore	Novelty	-0.3916	0.0599	132	-6.53	<0.0001
	Aurore	Oliver	-0.7891	0.0547	132	-14.42	<0.0001
	Bison	Novelty	-0.4677	0.0605	132	-7.74	<0.0001
	Bison	Oliver	-0.8652	0.0552	132	-15.67	<0.0001
	Novelty	Oliver	-0.3974	0.0485	132	-8.19	<0.0001

Appendix II: Summary of complete analysis of variance of row or column effect of modified augmented design based on plot and subplot controls, for seven traits in Aurore x Oliver recombinant inbred line population at Saskatoon (SK) and Morden (MO) wilt nurseries in 2013 and 2014, and the suggested adjustments. Method 1 (M1) based on design structure of plot control, and Method 3 (M3) based on covariance adjustment and Method 1+3, a combination of the above two.

Location	Year	Trait	Source	DF	SS	MS	F	P	Significance	Suggested adjustment method by ANOVA
MO	2013	AUDPC	Genotype	1	620944.00	620944.00	3418.54	<0.001	**	
			Row	5	289.47	57.89	0.32	0.897	ns	
			Column	5	1609.14	321.83	1.77	0.157	ns	
			Row x Column (Whole plot error)	24	4359.36	181.64	0.63	0.865	ns	Unnecessary
			Control	1	416198.30	416198.30	1442.10	<0.001	**	
			Subplot error	22	6349.31	288.61				
		Vigour 1	Genotype	1	39.06	39.06	411.59	<0.001	**	
			Row	5	0.87	0.17	1.83	0.145	ns	
			Column	5	0.70	0.14	1.48	0.234	ns	
			Row x Column (Whole plot error)	24	2.28	0.09	1.10	0.412	ns	Unnecessary
			Control	1	33.84	33.84	392.74	<0.001	**	
			Subplot error	22	1.90	0.09				
		Vigour 2	Genotype	1	61.36	61.36	110.45	<0.001	**	
			Row	5	1.81	0.36	0.65	0.664	ns	
			Column	5	1.81	0.36	0.65	0.664	ns	
			Row x Column (Whole plot error)	24	13.33	0.56	2.44	0.019	*	Method 3
			Control	1	37.50	37.50	165.00	<0.001	**	
			Subplot error	22	5.00	0.23				

Vigour 3	Genotype	1	93.44	93.44	178.62	<0.001	**	
	Row	5	2.33	0.47	0.89	0.502	ns	
	Column	5	3.67	0.73	1.40	0.259	ns	
	Row x Column (Whole plot error)	24	12.56	0.52	0.68	0.821	ns	Unnecessary
	Control	1	51.04	51.04	66.38	<0.001	**	
	Subplot error	22	16.92	0.77				
Wilt 1	Genotype	1	312.11	312.11	2106.75	<0.001	**	
	Row	5	0.33	0.07	0.45	0.809	ns	
	Column	5	0.00	0.00	0.00	1.000	ns	
	Row x Column (Whole plot error)	24	3.56	0.15	1.96	0.059	ns	Unnecessary
	Control	1	228.17	228.17	3011.80	<0.001	**	
	Subplot error	22	1.67	0.08				
Wilt 2	Genotype	1	336.11	336.11	2200.00	<0.001	**	
	Row	5	0.56	0.11	0.73	0.610	ns	
	Column	5	1.22	0.24	1.60	0.198	ns	
	Row x Column (Whole plot error)	24	3.67	0.15	1.15	0.371	ns	Unnecessary
	Control	1	222.04	222.04	1674.83	<0.001	**	
	Subplot error	22	2.92	0.13				
Wilt 3	Genotype	1	361.00	361.00	803.88	<0.001	**	
	Row	5	1.22	0.24	0.54	0.741	ns	
	Column	5	3.56	0.71	1.58	0.203	ns	
	Row x Column (Whole plot error)	24	10.78	0.45	0.58	0.905	ns	Unnecessary
	Control	1	228.17	228.17	292.41	<0.001	**	
	Subplot error	22	17.17	0.78				

SK	2013	AUDPC	Genotype	1	457877.80	457877.80	2294.06	<0.001	**	
			Row	5	2118.67	423.73	2.12	0.097	ns	
			Column	5	913.33	182.67	0.92	0.488	ns	
			Row x Column (Whole plot error)	24	4790.22	199.59	0.20	1.000	ns	Unnecessary
			Control	1	228540.20	228540.20	233.38	<0.001	**	
			Subplot error	22	21543.67	979.26				
		Vigour 1	Genotype	1	11.11	11.11	25.00	<0.001	**	
			Row	5	6.22	1.24	2.80	0.040	*	
			Column	5	3.89	0.78	1.75	0.162	ns	
			Row x Column (Whole plot error)	24	10.67	0.44	0.61	0.877	ns	Method 1
			Control	1	7.04	7.04	9.73	0.005	**	
			Subplot error	22	15.92	0.72				
		Vigour 2	Genotype	1	23.36	23.36	52.56	<0.001	**	
			Row	5	1.47	0.29	0.66	0.655	ns	
			Column	5	0.81	0.16	0.36	0.869	ns	
			Row x Column (Whole plot error)	24	10.67	0.44	1.10	0.416	ns	Unnecessary
			Control	1	12.04	12.04	29.71	<0.001	**	
			Subplot error	22	8.92	0.41				
		Vigour 3	Genotype	1	14.69	14.69	31.43	<0.001	**	
			Row	5	1.58	0.32	0.68	0.645	ns	
			Column	5	1.25	0.25	0.53	0.748	ns	
			Row x Column (Whole plot error)	24	11.22	0.47	1.30	0.270	ns	Unnecessary
			Control	1	12.04	12.04	33.46	<0.001	**	
			Subplot error	22	7.92	0.36				

		Wilt 1	Genotype	1	235.11	235.11	995.76	<0.001	**	
			Row	5	3.56	0.71	3.01	0.050	ns	
			Column	5	1.89	0.38	1.60	0.198	ns	
			Row x Column (Whole plot error)	24	5.67	0.24	0.41	0.981	ns	Unnecessary
			Control	1	145.04	145.04	253.58	<0.001	**	
			Subplot error	22	12.58	0.57				
		Wilt 2	Genotype	1	245.44	245.44	946.71	<0.001	**	
			Row	5	0.67	0.13	0.51	0.763	ns	
			Column	5	0.67	0.13	0.51	0.763	ns	
			Row x Column (Whole plot error)	24	6.22	0.26	0.30	0.998	ns	Unnecessary
			Control	1	104.17	104.17	119.57	<0.001	**	
			Subplot error	22	19.17	0.87				
		Wilt 3	Genotype	1	215.11	215.11	860.44	<0.001	**	
			Row	5	1.89	0.38	1.51	0.224	ns	
			Column	5	0.89	0.18	0.71	0.621	ns	
			Row x Column (Whole plot error)	24	6.00	0.25	0.49	0.956	ns	Unnecessary
			Control	1	112.67	112.67	218.71	<0.001	**	
			Subplot error	22	11.33	0.52				
SK	2014	AUDPC	Genotype	1	595984.00	595984.00	2127.25	<0.001	**	
			Row	5	1830.67	366.13	1.31	0.294	ns	
			Column	5	2257.33	451.47	1.61	0.195	ns	
			Row x Column (Whole plot error)	24	6724.00	280.17	1.29	0.277	ns	Unnecessary
			Control	1	401192.00	401192.00	1844.59	<0.001	**	
			Subplot error	22	4784.92	217.50				

Vigour 1	Genotype	1	44.44	44.44	168.42	<0.001	**	
	Row	5	2.22	0.44	1.68	0.177	ns	
	Column	5	2.89	0.58	2.19	0.089	ns	
	Row x Column (Whole plot error)	24	6.33	0.26	1.45	0.192	ns	Unnecessary
	Control	1	24.00	24.00	132.00	<0.001	**	
	Subplot error	22	4.00	0.18				
Vigour 2	Genotype	1	17.36	17.36	48.08	<0.001	**	
	Row	5	3.81	0.76	2.11	0.099	ns	
	Column	5	1.81	0.36	1.00	0.439	ns	
	Row x Column (Whole plot error)	24	8.67	0.36	0.96	0.538	ns	Unnecessary
	Control	1	18.38	18.38	49.00	<0.001	**	
	Subplot error	22	8.25	0.38				
Vigour 3	Genotype	1	14.69	14.69	33.41	<0.001	**	
	Row	5	3.58	0.72	1.63	0.190	ns	
	Column	5	3.92	0.78	1.78	0.155	ns	
	Row x Column (Whole plot error)	24	10.56	0.44	0.77	0.735	ns	Unnecessary
	Control	1	18.38	18.38	32.13	<0.001	**	
	Subplot error	22	12.58	0.57				
Wilt 1	Genotype	1	354.69	354.69	1126.68	<0.001	**	
	Row	5	2.25	0.45	1.43	0.250	ns	
	Column	5	2.25	0.45	1.43	0.250	ns	
	Row x Column (Whole plot error)	24	7.56	0.31	1.73	0.100	ns	Unnecessary
	Control	1	216.00	216.00	1188.00	<0.001	**	
	Subplot error	22	4.00	0.18				

Wilt 2	Genotype	1	342.25	342.25	2240.18	<0.001	**	
	Row	5	1.58	0.32	2.07	0.104	ns	
	Column	5	1.25	0.25	1.64	0.189	ns	
	Row x Column (Whole plot error)	24	3.67	0.15	0.94	0.563	ns	Unnecessary
	Control	1	234.38	234.38	1438.95	<0.001	**	
	Subplot error	22	3.58	0.16				
Wilt 3	Genotype	1	300.44	300.44	754.60	<0.001	**	
	Row	5	1.67	0.33	0.84	0.536	ns	
	Column	5	1.33	0.27	0.67	0.650	ns	
	Row x Column (Whole plot error)	24	9.56	0.40	1.22	0.320	ns	Unnecessary
	Control	1	228.17	228.17	700.42	<0.001	**	
	Subplot error	22	7.17	0.33				

Appendix III: Genotype sequencing of a subset of 178 lines of Aurore x Oliver RIL population with next generation (Illumina) sequencing (NGS)

Determining the location of wilt resistance genes/ QTL in a flax genome can be beneficial to develop wilt resistant cultivars and to reduce the time in breeding programs. The AO RIL population was genotyped by NGS to be used in a QTL analysis. However, 99.9% of the data showed segregation distortion.

Methodology

Seeds of the 200 lines were sent to Agriculture and Agri-Food Canada (AAFC), MB, where they were grown and tissue samples collected from seedlings for DNA extraction. Of these, 178 RILs were sequenced by NGS at the BC Cancer Agency. Illumina sequence reads of Aurore and Oliver were aligned to the whole genome assembly of CDC Bethune (the reference genome) and the data were assembled by a pipeline (by Dr. Frank You) to identify single nucleotide polymorphisms (SNPs) of Aurore, Oliver and the RILs. Only the assembled SNP data files and marker descriptions were provided.

Results

The resequence data included ≈22,000 SNP markers (minor allele frequency (MAF) ≥ 0.02), of which all except seven, deviated from the 1:1 ratio expected for a RIL population. Therefore, the sequence data was reanalyzed using the same pipeline with CDC Bethune as the reference genome. Again, of ≈23,000 SNP markers (MAF ≥ 0.02), only three markers were observed to segregate in a 1:1 ratio, which indicated almost complete segregation distortion.

Hence, the markers were separated by their linkage groups and the number of markers segregating towards one parent in each linkage group was counted. Next, a chi squared test was performed for each linkage group to test if the markers within a linkage group were distributed in a 1:1 ratio (Table 5.3).

Table A Single nucleotide polymorphism summary and chi square value by linkage group, for the flax cross Aurore by Oliver. The number of SNPs in each linkage group indicate similarity of each RIL with either parent.

Linkage group	Number of SNPs with majority of the RILs same as Aurore	Number of SNPs with majority of the RILs similar to Oliver	Total no. of SNPs	Chi square	P value
1	1373	375	1748	569.80	<0.0001***
2	1870	131	2001	1511.30	<0.0001***
3	1382	526	1908	384.03	<0.0001***
4	374	399	773	0.81	0.37
5	846	399	1245	160.49	<0.0001***
6	659	402	1061	62.25	<0.0001***
7	1447	101	1548	1170.36	<0.0001***
8	402	418	820	0.31	0.58
9	348	675	1023	104.52	<0.0001***
10	809	136	945	479.29	<0.0001***
11	408	624	1032	45.21	<0.0001***
12	3272	383	3655	2283.54	<0.0001***
13	570	648	1218	5.00	<0.0001*
14	486	1264	1750	345.88	<0.0001***
15	408	755	1163	103.53	<0.0001***

*P<0.05, **P<0.01, ***P<0.001

SNPs associated with two linkage groups (four and eight) were observed to segregate in a 1:1 ratio, while the SNP markers in the remaining linkage groups showed preference towards one parent or the other. Most of linkage groups displayed preference towards Aurore, while preference to Oliver was observed in linkage groups 9, 11, 13, 14 and 15.

Discussion

The SNP data of the AO population was first analyzed with the chi-square test to determine segregation. In a homozygous population, such as the RIL population used in

this study, the segregation of the two variants was expected to be 1:1. However, the population showed severe segregation distortion (99.9%), with less than ten markers displaying a 1:1 ratio. Therefore, QTL mapping was not conducted, since a high level of segregation distortion affects QTL detection with dominant effects (Xu, 2008) and can cause errors in preparation of a linkage map by affecting the marker distance and segregation distortion loci estimations (Li et al., 2015). The sequence data were separated by linkage group to determine if one parent dominated at each linkage group. There was no dominant parent for linkage groups 4 and 8, while the RILs showed much more inheritance from Aurore in linkage groups 1-3, 5-7, 10 and 12, and from Oliver in linkage groups 9, 11, 13-15.

The degree, direction and genetic effects of segregation distortion depends on a few factors such as the species, crosses, mapping population type and marker technology used (Liu et al., 2010). Segregation distortion regions (SDR) have been identified in several crops, where the distorted loci are clustered together. An F_2 and a DH population developed from the same cross of two *japonica* rice cultivars (*Oryza sativa* subsp. *japonica*) displayed 7% and 19% distortion respectively and the distorted regions differed between the populations (Yamagashi et al., 2010). High segregation distortion in RIL populations compared to F_2 populations, have been suggested to be due to natural selection of many generations and artificial sampling (Liu et al., 2010). Segregation distortion has also been reported to be higher in interspecific populations than in intraspecific populations, although higher marker polymorphism between parents and reduced or selective fertility might be responsible for this (Liu et al., 2010).

Molecular marker analysis is an efficient method of determining segregation distortion. However, the degree of distortion observed may differ based on the type of marker used. Lorieux et al. (1995) reported that the estimation of recombination fractions by codominant

markers was less affected by selection, and the dominant markers provided less information when there was segregation distortion. They determined, seed fertility genes and gametophytic competion genes caused this distortion. Thus, natural selection through gametophytic selection of one parent or seed sterility, the population type and size, type of marker technology used and parents used in the cross, can determine the amount of segregation distortion observed.

Segregation distortion observed in the AO RIL population in this study, was so extreme that none of the above causes can completely explain it. One hypothesis may be that there was selection through the generations. However, the RILs were not observed to carry the phenotype of one parent, thus contradicting this hypothesis. Also, when the population was screened using ten SSR markers, a 1:1 ratio was observed (personal communication Dr. John Paul Trouve, Terre de Lin, France). Another possible explanation is that there was an error in SNP calling, using CDC Bethune as the reference genome, given that CDC Bethune is a Canadian cultivar and Aurore and Oliver are French cultivars. Also, there could be an error with the pipeline that was used to assemble the SNP data. While the same pipeline was previously used successfully, to assemble the data for Canadian flax populations, the assembled data for another French RIL population also showed extreme segregation distortion (personal communication Dr. Frank You, AAFC, Morden, MB, and Dr. John Paul Trouve).

Appendix IV: Screening a subset of 92 lines of Aurore x Oliver RIL population with four simple sequence repeat (SSR) markers

Due to segregation distortion, the SNP data of the AO population, was not useful for construction of a genetic map or conduct quantitative trait analysis. Therefore, four SSR markers previously identified to be associated to wilt were used to screen 92 selected RILs (Lu2044, Lu2184a, Lu2230 and Lu2612). Being codominant markers, SSR markers can be beneficial in determining the frequency of both alleles, though they provide less coverage of the genome in comparison to SNPs.

Methodology

Four simple sequence repeats (SSR) markers (Lu2044, Lu2184a, Lu2230, Lu2612), that were previously identified to be associated with wilt in the flax core collection by Dr. Sylvie Cloutier (AAFC, Winnipeg, MB) in a marker-trait association study, were used to identify polymorphisms between the parents Oliver and Aurore (Table 5.1).

DNA was extracted from the leaves of 92 RILs, which varied in disease resistance /susceptibility based on the phenotypic screening results. Although only SSR makers Lu2044, and Lu2612, were identified to be polymorphic, all four markers were screened.

Table B Simple sequence repeat markers identified to be associated with flax wilt, position in the flax genome and polymorphism between parents (Aurore and Oliver) of the RIL population, used in genetic screening of 92 RILs with varying levels of resistance to flax wilt

Marker	Linkage group	Scaffold	Location	Polymorphism
Lu2184a	LG1	1202	258323 – 288882	No
Lu2044	LG3	471	274194 – 274345	Yes
Lu2230	LG4	272	1555447 -	No
Lu2612	LG12	25 or 140 (duplicated region)	521375 (25) or 427300 (140)	Yes

Leaf tissues of Oliver and Aurore (≈100 mg) were snap-frozen in liquid nitrogen and lyophilized. Genomic DNA was extracted with the DNeasy plant kit (Qiagen, Missisauga, ON, Canada) according to the manufacturer's instructions and was quantified using fluorometry with Aurore ≈12 ng/ml and Oliver ≈20 ng/ml.

Crude extraction was carried out to obtain the DNA from the 92 RILs, by grinding a 2-3 mm long frozen leaf tip in 40 µl of NaOH in a deep well plate. Next it was heated at 94 °C for 45 sec followed by the addition of 60 µl of Tris buffer. Finally, the plate was heated at 94 °C for 3 min.

One microliter of genomic DNA from each parent was used as template for SSR amplification by polymerase chain reaction (PCR) with a final volume of 12 µL per reaction with M-13 labelled FAM added for identification of PCR products. PCR was programmed for three minutes at 94°C, followed by 20s at 94°C. Next, there were four cycles with a temperature gradient from 56 °C to 50 °C at 2 °C variation each lasting 30 sec and each temperature point followed by 30 sec at 72 °C and 20 sec at 94 °C. This was repeated three times followed by 29 cycles with 20 sec at 94°C. Electrophoresis of the final PCR product continued successful amplification. The sample was then it was stored at -20°C.

The PCR product was diluted ten times in formamide to obtain a total volume of ten microliters which was then mixed on the shaker and centrifuged. Finally, it was heated to 94 °C for 10 min. The sample was analyzed with a genetic analyzer (Applied Biosystems). Output files were analyzed by Genemapper and the fragment sizes were estimated using the internal size standards and for each RIL.

Results and Discussion

Normal segregation of the RILs was observed only with Lu2044. Forty-eight of the RILs had fragment sizes the same as Aurore (resistant parent) of 176 bp and 212 bp, while

34 of the RILs had fragments at 168 bp and 204 bp, corresponding with Oliver. There were seven RILs with missing data and three RILs with fragments corresponding to both parents, indicating heterozygosity. The segregation of the RILs for marker Lu2044 was non-significant, thus in accordance with 1:1 ratio expected of a RIL population (χ^2 = 2.39, p=0.12). When the ANOVA was calculated for the two alleles, using the phenotypic data from the wilt nurseries and controlled conditions, it was not significant. This suggested, that the marker was not associated with wilt resistance in this population.

Lu2612, the only other marker for which the parents were polymorphic, produced the same sized fragment as Aurore (294 bp), for all RILs except for RIL11922 which had a fragment of 281 bp. There were 16 RILs with missing data. While these markers have been previously identified to be associated with wilt resistance in the flax core collection, they were not associated with wilt resistance in the AO population.